

3 January 1980

# Nuclear power: the critics must be heard

WHEN announcing the UK government's decision to build at least one nuclear reactor per year during the decade beginning 1982 (see page 3) Mr David Howell, Energy Secretary, placed great emphasis on the need for safety.

After the accident at the Three Mile Island nuclear power plant in Pennsylvania, US last March, public fears about the safety of nuclear power are justifiably strong. And in the UK, that fear is likely to be heightened by the government's probable intention to base the new programme on the pressurised water reactor — of a similar type to that at Three Mile Island — rather than the UK developed advanced gas cooled reactor.

Mr Howell was therefore right to stress the need for safety and to reaffirm the government's intention of holding a public inquiry on the safety of the PWR before the first is built. This view is endorsed by the new House of Commons Select Committee on Energy which recently announced that the PWR is to be its first topic for study. It is to call on Mr Howell and representatives from the nuclear industry and the nuclear opponents as witnesses and is to pay special attention to safety.

However, what will be most important over the next two years leading up to the public inquiry and indeed, throughout the subsequent construction of any power stations, is that Mr Howell's promise is seen publicly to be adhered to. The need for the nuclear industry to be entirely open about its plans is greater now than it has ever been. This is not the time for it to hide behind the government's decision in the hope that it will be out of the limelight. More than ever before, public attention will be turned on it and the public will expect its opinions to be taken into

account.

The major lesson which must be learned from previous nuclear debates in many countries is the need for the critics of nuclear power to have complete access to information. We endorse the government's decision to release unabridged safety reports on all nuclear power stations but would go one step further. We recommend that the Nuclear Installations Inspectorate consider the opinions of the serious nuclear critics during any future safety assessments of nuclear power stations. This means that the critics must have the same rights of access to design and safety documents and the same rights to make site inspections as do NII inspectors.

Greater openness will not be to the detriment of the nuclear industry. On the contrary, an industry which is seen publicly to be taking the right decisions about safety is one which will receive support at home and orders from abroad. Where safety is concerned, the industry should not worry about the need for commercial secrecy because the industry which is the most successful in demonstrating the safety of its reactors will be the one most successful in selling them.

Finally, the current decision should not automatically lead to the first step along the road to a major commercial fast breeder reactor programme. Rather it should be seen as a way of keeping our options open well into the 1990s. In the meantime, research — and particularly the development — of alternative sources of energy and new uses for coal should be stepped up. Only after equivalent funding will it be possible to make a balanced judgement between — say — fast breeders and wave power. □

## David Davies' editorship ends

FOR six and a half years *Nature*, and much of the world's scientific community, has enjoyed Dr David Davies' work as its editor. A man of character, he has impressed his personality and leadership on the journal. A firm believer, as I am, that the specifically human characteristic of adaptability requires change to express itself, to lead to the maximum contribution and real job satisfaction, he has always lived by his precept.

Once again, he felt that the time had come to move on to a radically different activity. He has become director of the newly established northern centre for the Dartington Trust, at Barnstaple, Devon. Much as we will all miss him from his editorial desk, his new task of nurturing vitality and amenities in the rural parts of North Devon will certainly give full scope to his drive and creative thinking.

He came to *Nature* from work as a geophysicist in MIT's Lincoln Laboratory in the US, with particular concentration on the intricate field of the seismic discrimination and the detectability of underground nuclear explosions — efforts continually central to the much hoped for complete test ban treaty. He had come to geophysics from physics at Cambridge University.

He threw himself into his new tasks at *Nature* with characteristic flair and drive. His international outlook, the result of much travel and experience of work in two countries,

was translated into efforts which matured into a remarkable success of making *Nature* a journal concerned with the progress, health, organisation and vitality of science in all countries of the world. We should perhaps be particularly grateful for the coverage of countries of the developing world, but his own intimate knowledge of the United States scene, his selection of correspondents in Canada and on the European continent, his ensuring the reporting of what goes on in the USSR and Eastern Europe, all this has led to a global outlook of educational benefit to all *Nature's* readers.

Personally, I have most enjoyed his editorials, pithy, hard-hitting and relevant. One may not have agreed with every one of them, but none was dull. But the journal's success is above all based on the original science it contains. It was no mean effort to have kept and increased its attraction to scientists everywhere for the rapid but responsible publication of relevant new advances. To have added to this central core of original letters a series of important and novel review-type articles in every issue has materially added to the value of the journal. The book reviews too have been a source of much enjoyment and profit to me.

His six and a half years with *Nature* have made an impact on all its readers. We all wish him well in his new and very different tasks.

Hermann Bondi



## United States

# NAS calls for global aerosol ban

An international ban on non-essential uses of chlorofluorocarbon (CFC) aerosol propellants is now the most effective way to reduce the dangers to health and food supplies from further depletion of the ozone layer — says a report published by the National Academy of Sciences. **David Dickson** reports.

THE US banned aerosol spray cans a year ago, and this has led to a 50% drop in the use of CFCs.

The report points out that there remain many other uses of CFCs — for example in automobile air conditioning units, or in the production of flexible plastic foam cushioning — equally in need of control. Such non-propellant emissions could bring the total CFC emissions back up to previous levels within seven to ten years.

Reducing such uses, however, is likely to be difficult, since in many cases there are no acceptable substitutes. But the report points out that progress could be achieved if other industrialised nations would agree to a ban on non-essential aerosols: "should all countries decide to take action comparable with that already taken by the United States and Sweden, between one third and one half of the world's present CFC released would be avoided.

"Such a reduction would be a great step forward in decreasing the threat to the world's food supply, even though the magnitude of this threat is uncertain, and its advantages would far outweigh the relatively small costs of substituting alternative propellants and devices."

The report was prepared by the Committee on Impacts of Stratospheric Change and the Committee on Alternatives for the Reduction of Chlorofluorocarbon Emissions of the Academy's National Research Council. It was commissioned by the US Environmental Protection Agency.

Its strong message adds further fuel to the controversies that have been building up in other industrialised countries since the hypothesis that CFC emissions could seriously deplete the ozone layer — subsequently increasing the exposure of humans, animals and plant-life to harmful ultra-violet radiation — was first raised by US scientists in 1974.

Two months ago, another academy committee confirmed earlier conclusions that, if emissions continue at the present rate, the amount of ozone in the atmosphere is likely ultimately to be depleted by about 16%, half taking place within the next 30 years.

A few countries, in particular Sweden and Norway (expected soon to be joined by Canada) have agreed to follow the US example. But West Germany, France, the UK, Japan and Italy (who, together with the US, account for 90% of the West's production of CFCs) have refused to

impose stringent controls.

There are signs that their position may be shifting. Member countries of the European Economic Community, for example, which earlier this year agreed voluntarily to reduce non-essential aerosol usage by 30% by 1982, last week agreed to advance the date by six months, and to hold back on additional investment in industrial uses of CFCs.

The US however, would like things to move much faster. The report recommends the complete elimination of non-essential aerosols on a global basis. Dr John W Tukey, of Bell Laboratories, chairman of one of the panels, said last week: "The US has done it, and it wasn't very painful."

The new report examines in detail some of the consequences of the depletion in the ozone layer predicted by the companion study. And it discusses some of the alternative technologies, such as sealed air conditioning units, that might reduce or replace CFC-emitting processes.

The most obvious human health effect, it says, would be a further increase in skin cancer, (melanoma), already one of the most rapidly increasing forms of cancer in the US. The report predicts several thousand more cases of melanoma a year,

## Heavy water reactors are less proliferation resistant

Although making more efficient use of uranium, heavy water reactors are less proliferation resistant than either high temperature gas-cooled reactors or light-water reactors, according to the draft report of the US Department of Energy's Non-Proliferation Alternative Systems Assessment Programme. The report, published last week in Washington for public comment, suggests that AGRs might have an application in the industrial process-heat market. In general, however, it suggests that the US can minimise the risks of proliferation by pushing ahead with the development of LWRs, and with liquid-metal fast breeder reactors. "In order to influence the liquid-metal fast breeder reactor programs of other countries, not only must the US be a member of the fast-breeder development community, but it also needs to exercise leadership in developing the technological measures which would reduce the proliferation risk of fast breeder reactors," the report says.

"of which a substantial fraction would be fatal."

But the committee expresses greatest concern over the effect on global food supplies. "Here we know enough to be concerned, but not enough to predict what the biological effects would be," says Dr Tukey.

As DNA is affected by exposure to solar ultra-violet radiation the effect on the growth and reproduction of animals and plants would be expected to lower the yields of farms and fisheries.

Experiments indicate that some crops, such as sugar beets, tomatoes and corn, might be "significantly affected" by a 16% ozone depletion.

The report points to the particular threat to aquatic food systems studies of the UV sensitivity of young anchovies indicate that this commercially-important food source now lives near the limit of its solar-UV tolerance when at the surface of the water, as do certain crab and shrimp larva. "A 16% reduction in ozone concentration would be distinctly harmful to them in a surface location," it says.

Discussing the non-aerosol uses of CFCs, the report says that in many cases alternative technologies already exist, in particular for the agents used in blowing flexible plastic foam, and for the solvents used for cleaning metal or for sterilising medical supplies.

In other cases, however, controlling the use of CFCs is not likely to be so simple. It picks out their use in automobile air conditioning systems, the largest single sources of CFCs remaining in the US.

The prospects of reducing emissions from this source are "not good", though modifications to minimise leakage could yield significant reductions without burdensome costs.

The report outlines a variety of measures to restrict non-aerosol uses, from a complete ban on certain applications — the simplest approach, but "not always advantageous" — to a judicious mixture of tax measures, quotas on specific uses, and public education.

The ozone problem differs from earlier environmental threats, since wherever CFCs are emitted, the ultimate effect is global. It is therefore the forerunner of another serious problem: the increased atmospheric concentration of carbon dioxide. It concludes: "the nations of the world collectively need to devise mechanisms for dealing with problems of this sort, assessing the scientific evidence as it develops and finding ways to agree on effective control actions, since these are not problems that can be adequately dealt with by the actions of only a few nations."



## Italy

# Confronting the Euro agencies

**Vito Scalia, Italy's science minister, alerted *Nature* shortly before Christmas (see 20 December p 768) to the three-pronged attack he was to make on European research organisations. Speaking in the room in the Rome convent where Galileo was tried (right) Scalia demanded that Italy get fairer treatment from ESA, that CERN delay its decision on a new Director-General, and that the EEC adopt an experiment on nuclear reactor meltdown. The results of his efforts are now becoming plain, as **Robert Walgate** reports.**



ITALY'S representatives at CERN, the European organisation for subnuclear research, withdrew from the 19 December Council meeting and refused to endorse the appointment of German Professor Herwig Schopper, as new Director-General from 1 January 1981.

The Italians are thus carrying out their threat to "consider seriously dissociating" Italy from CERN. One of the present joint CERN Directors-General, Dr John Adams, offered to visit Rome to mediate in the dispute — which is principally between Italy and Germany — but Rome refused.

The delegation is apparently still smarting from the rebuff it received — at a preliminary meeting — to its proposal that the future programmes of CERN should be "better clarified" before an appointment is made. Italy fears that Germany is not

altogether sincere in its commitment to CERN's future, as it is considering a proposal for an accelerator (HERA) that Italy believes is competitive with CERN's LEP (see page 5).

The Italian government has its own candidate for the CERN Director-Generalship, Professor Antonino Zichichi. But Italian scientists working at CERN were nervous of their delegation's tactics, as they rely on CERN and other non-Italian laboratories for their livelihood.

## JET under threat

THE European Commission's Super Sara experiment on nuclear reactor meltdown is now supported by eight of the nine community members, with only France objecting.

At a meeting in Brussels of energy ministers of the nine, Italy — the strongest supporter of Super Sara — swung a number of other objectors (including Britain) behind her. But Pierre Aigrain, France's Minister of Science, continued to hold strong objections.

France claims that its similar experiment at Cadarache is more advanced than Super Sara — which would be conducted at the EEC Joint Research Centre at Ispra, north Italy, on an isolated circuit within the reactor ESSOR.

France's objections have led Italy to act on its threat to hold up once again the EEC's 1980-85 budget for energy research.

## ESA absorbs objections

ITALY'S attack on European research organisations succeeded in one respect: a tacit agreement of the Council of the European Space Agency to adjust the budget in Italy's favour to the tune of 27 million units of account (mua) (about \$40 million).

Italy had argued that it had lost some 32 mua over the allocation of contracts to build Spacelab, the orbiting laboratory which will be flown by the Space Shuttle.

The adjustments should be confirmed at a meeting on 23 January. They will come in the form of a reduced contribution over four years, and a greater allocation of Spacelab follow-on projects. □

## United Kingdom

# Government plumps for PWR power station

THE UK government has decided to embark on a major new nuclear programme. At least one nuclear power station is to be built each year during the decade beginning 1982 and the nuclear industry is to be revitalised to cope with the demands to be made of it. The estimated cost of the programme — to be paid for by the Central Electricity Generating Board — is £10-12 billion at 1979 prices. It is expected to increase the nuclear contribution to electricity supply from 12% to over 30% by the early 1990s.

Mr David Howell, Secretary of State for Energy, said when announcing the decision that the UK needed a nuclear programme of this size if it was to maintain the type of society it has now into the 1990s, when North Sea oil and gas production would decrease. The size of the programme, equivalent to about 15,000 MW, was based on Department of Energy forecasts of energy demand, assuming a 20% saving in consumption from conservation, he said.

A substantial programme was also needed to build up and maintain a strong

industry; the lack of orders over the past decade had led to its decline. "If we are to reverse this trend and ensure that the industry is on a sound footing, we must act now", said Mr Howell. Under the reorganisation planned for the industry, the National Nuclear Corporation (NNC) and the Nuclear Power Company are to be brought under the umbrella of one board. The new NNC will then have total responsibility for management of all nuclear projects.

One of its first tasks will be the design of a pressurised water reactor under license from Westinghouse. In line with the previous Labour Government's decision, the Conservatives intend that the first power station of the programme will be a PWR. The design must be approved for safety by the Nuclear Installations Inspectorate and must be subject to a public inquiry. The NNC estimates that it could be ready for this by the end of 1981, and that construction could begin in 1982.

The NNC is also to assume responsibility for managing the UK's advanced gas

cooled reactor (AGR) programme. By building two more AGRs as well as one PWR, the CEBG hopes that the final choice of reactor can be left for some time. However Mr Howell has said that a commitment to one system or the other would have to be made fairly early on in the programme.

The major advantage of the PWR over the AGR is capital cost. The CEBG estimates that the capital cost per MW is £750,000 for an AGR and £680,000 for a PWR.

● The British nuclear industry and the NII have released preliminary statements on the implications of the accident at Three Mile Island. They conclude that the recommendations of the President's commission, set up to investigate the accident, do not apply to the UK. Fault was found with the regulatory procedure in the US and the standard of operator training but not with the basic PWR design. Most of the suggestions for improvement, they say, are already incorporated in the UK regulatory procedure.

**Judy Redfearn**



## Soviet Union

# Brezhnev counts on Academy of Sciences to organise applied research

LAST month, the Soviet Academy of Sciences met to discuss the role of scientists in solving the urgent problems facing the national economy. This was on instructions from the Party Central Committee and the government, and was a response to Mr Brezhnev's November address to the Central Committee. In his swingeing attack on Soviet planning and production, Brezhnev singled out for castigation a number of ministries (including railways, power and ferrous metallurgy), the State Planning Committee and the "bureaucratic and local interests" which "weaken the strength of the plan". In tackling the "vital tasks", both of production and planning, said Brezhnev, the Party counts on the active assistance of scientists, the USSR Academy of Sciences, the Republic and branch academies and all scientific research institutes. "The State Committee for Science and Technology", he added, "must also work in a more energetic manner".

This is not the first time that Brezhnev has called on the academy for assistance. At the Twenty-Fifth Party Congress in 1976, he had declared that the academy was to act as chief coordinator of all scientific work in the country. This caused some confusion, not least to the academicians themselves. It now seems certain, however, that what Brezhnev meant was that the academy should coordinate fundamental research throughout the USSR, stressing the need to implement the practical applications of such research.

At the same time, a number of specialists from the academy were involved (together with officials of the State Planning Committee, the relevant ministries and the State Committee for Science and Technology) in working out a "complex programme" for the development of science and technology up to 1990. Work on this programme began in 1972, under the general direction of a special commission of the academy presidium, but by summer 1979 it was still not completed. In the course of work on the "complex programme", there have been numerous set-backs; perhaps the greatest being the change from forecasts for individual technologies with little coordination between them (some 150 in the first drafts) to 25 "integrated" programmes, 17 for science and technology and eight for social questions, covering broader fields such as energy, health, industrial materials, and demographic trends.

How far existing plans for the "complex programme" will be affected by the latest appeal to the academy is not clear.

Addressing the assembly, the President of the academy, Anatolii P. Aleksandrov stressed the Soviet Union's continuous shortfall of ferrous metals, cement and fertiliser, the need to pay greater attention to the "economic utilisation" of energy, raw materials and agricultural produce.

Nikolai Baibakov, Chairman of the State Planning Committee and a Deputy Chairman of the USSR Council of Ministers, stressed the need for a long-term comprehensive programme for transport, including pipelines for pulp, pipelines using containers, magnetic suspension transport and linear motor trains. The automation of manual labour (a major problem for the Soviet Union with its falling demographic trend) was also stressed. Baibakov commended the academy's current research on energy-saving types of equipment and new types of polymer, which, he said, should ultimately save the economy some 1000 million roubles.

Media comments on the session, presumably from official releases, stressed the need for a new comprehensive plan for science and technology up to the year 2000, giving priority to new regions in the far north, Siberia, and the Soviet Far East.

Setting the tone for such developments, Baibakov noted that "socialist planning has always been 'target-orientated'", citing Lenin's plan to electrify the whole country, the construction of heavy industry in the 1930s, the Virgin lands scheme of the early 1960s, and the current construction of the Baikal-Amur Mainline railway.

His remarks have some suggestion that science and technology might take on the status of a "crash programme". (This would not be out of key with the economic "mini-reform" proposed last July). Aleksandrov, the President of the Academy has, incidentally, considerable experience of this type of priority development (nuclear research) as had his predecessor Mstyslav Keldysh (space). Such crash programmes have been particularly successful in sectors with some defence connections (since defence falls outside the main planning structure). Any attempt to introduce wide-scale crash planning would, however, appear to be counter-productive — there would be too many priorities competing for too few resources.

The main weakness of Soviet science planning, however, remains the point at which production takes over from research. Since the mid 1960s the planners have set considerable store on closer links

between research and academic institutes on the one hand, and local farms or factories on the other. The classic case of such cooperation is the Siberian branch of the academy at Novosibirsk. Here a network of research institutes, design centres and experimental production factories has been set up. These are maintained by, and officially subordinate to, the relevant ministries, but the various institutes of the Academy of Sciences are responsible for the scientific leadership.

Recently, a number of *ad hoc* "science centres" were established throughout the Soviet Union, often simply by formally linking existing research and industrial establishments. Many of these centres, Baibakov told the academy, are "observed to be dissipating their resources" and wasting time on "second grade problems", which are not "in the mainstream of technical progress". As a result, "many ministries (presumably those censured by Brezhnev) have been unable to fulfil their plans for science and technology".

Since the Siberian branch of the academy was established, a number of academicians, including its chairman academician G I Marchuk, have pointed out that the dual control system could lead to precisely this kind of dissipation. However, this has always been presented as a danger. By concentrating too much on the minutiae of day-to-day problems, the ministry could distract researchers and resources from basic and far-reaching tasks. Academician B Paton of the Ukrainian Academy of Sciences would prefer that even the design and pilot-production facilities should be subordinate to the academy, so as to obviate this hazard.

Baibakov's remarks, however, seem intended to exonerate the ministry planners and, perhaps, by implication, shift a greater responsibility to the academics. The relationship between the Academy of Sciences (traditionally orientated to fundamental research) and the state committee for Science and Technology, with its stress on production is always a delicate one. Although Brezhnev has censured the state committee and called once again on the academy's assistance, the state committee has recently taken over a duty which would normally be considered the prerogative of the academy. At the forthcoming Hamburg "scientific forum", where most of the Helsinki signatories will be represented by their academies of science, the Soviet delegation will be headed by the Chairman of the State Committee.

Vera Rich

# NEWS IN BRIEF

## CERN discloses plans for new accelerator

LEP, a particle accelerator to collide 22-130 GeV electrons with their antiparticles, positrons, at the same energy, would begin construction near Geneva in early 1982 if plans announced at last week's CERN Council meeting are accepted. John Adams, Director-General of the Centre for European nuclear physics (CERN), said that he planned to submit a firm proposal for LEP to the Council — the supreme body of CERN on which all member states are represented — in June 1980. A decision could be taken in June 1981.

LEP would probably be 5 km in radius, and be built underground. It could be built without increasing the present CERN budget of 593 million Sw Fr per year, and no new staff would be needed, said Adams; but the intersecting storage rings (for proton-proton collisions) would be phased out, and the old 8 GeV synchrocyclotron — CERN's first machine — reduced to serve only the low energy physics 'Isotope separator on line' (ISOLDE), which is widely used by Scandinavian members of CERN and has produced much useful data on short-lived isotopes.

## USSR to license use of advanced energy technologies

THE Soviet Union will license the use of 30 processes for eliminating the high sulphur content of coal and for developing electricity production through magnetohydrodynamics to the US. The agreement signed by the USSR foreign trade organisation Licensintorg and the US firm Control Data Corporation, is a consequence of a deal made several years ago when Control Data supplied a superconducting magnet to the USSR for use in MHD experiments. In return, Control Data saved the need to build a MHD test plant of its own.

## US to stop funding labour-saving agriculture research

THE US Secretary of Agriculture Mr Bob Bergland, has announced that federal research funds distributed to land-grant colleges and universities can no longer be used to support research into the mechanisation of agricultural processes leading to reduced employment for farm workers.

Such research has come under increasing criticism in recent years for contributing to unemployment among farmworkers, particularly among migrant workers who had previously been dependent on seasonal agricultural work for a major proportion of their income. Critics charge that the

federal research funds which are distributed to land-grant colleges on a pro-rata basis under the terms of the Hatch Act are merely serving to enrich large food-producing corporations.

However, Secretary Bergland's move was criticised by Dr Charles E Hess, head of the College of Agricultural and Environmental Science at the University of California, Davis, who said that "to suggest halting research which significantly contributes to enhancing agricultural productivity and efficiency is irrational and irresponsible."

## France and China sign agreement on geological research

AFTER eight days of meetings and visits to Chinese laboratories, Pierre Aigrain, the French Secretary of State for Research, signed an agreement on 14 December with Deng Xiao-ping, the Chinese vice-Premier, to provide for Franco-Chinese cooperation in geological research. The agreement gives the go-ahead to three French missions to study the north slope of the Himalayas in 1981, 1982, and 1983. In return France will provide laboratory equipment for Chinese geological research. Other areas of possible cooperation are teacher exchanges, the evaluation by French specialists of Chinese mineral reserves, particularly chromium, and the establishment of a Chinese geological information bank. Mr Deng Xiao-ping called the agreement a step in the direction of correcting the "loss of scientific talent due to the Cultural Revolution".

## Ariane makes it to the top

ARIANE, Europe's — but mostly France's — very own space launcher, went aloft on Christmas Eve (24 December) only 2 km away from its planned trajectory. A few days after the launch, measurements showed that the payload — a small test satellite — was in an orbit with perigee (closest approach to Earth) of 202.6 km, apogee (furthest distance) 36,019 km, with an inclination of the plane of the orbit to the equator of 17°.

The launch appeared to demonstrate that the hitches which caused the abandonment of two launch attempts within a week were indeed minor. They are attributed to small instrumentation malfunctions.

The greatest achievement — and surprise — of the launch was the perfect operation of the hydrogen-burning third stage, which had not yet passed its ground qualification tests.

European Space Agency press announcements before the launch had hedged around what would be considered a

'successful' launch, urging journalists to count mere ignition of the third stage a 'success'. There will be three more trials in 1980; one more 'success' will be considered a 'qualification' of Ariane for its first commercial launches in 1981.

## GMAG invites proposals for scale-up

THE UK Genetic Manipulation Advisory Group has issued guidelines for work with recombinant DNA "involving volumes of 10 litres or more". All proposals for "scale up" will be judged on the basis of a site visit as such will be considered case by case rather than by a set of strict rules. GMAG will examine the biological aspects of proposals for "suitable levels of disablement and containment".

Physical plant will also have to be examined for pressure safety, leaks or cracking and safety and emergency procedures will be inspected as will sterilisation provisions for waste products. A GMAG official said that the requirements are not beyond industry's present procedures for biological manufacture as for example in vaccine and antibiotic productions. "We want to encourage not discourage applications. Industrial procedures are usually much safer than laboratory work and if the proposals are consistent with safety we would be quite happy".

## Saccharin confirmed as weak human carcinogen

STUDIES carried out by the US National Cancer Institute have shown that heavy users of artificial sweeteners, especially those who consume diet drinks and other sugar substitutes, have a 60% increased risk of bladder cancer.

The study was based on interviews with more than 3000 bladder cancer patients and 6000 healthy people. It revealed "no increased risk" in the overall population being studied, but that those who used six or more servings a day of the sugar substitute saccharin, or two or more eight-ounce diet drinks, increased the risk of bladder cancer by 60%.

Following announcement of the results of the study, the Food and Drug Administration, whose attempt to ban the use of saccharin has been blocked by Congress, announced that it confirmed its previous contentions that artificial sweeteners posed a cancer risk to humans. Dr Jere E Goyan, Commissioner of the FDA, said that the study was consistent with previous animal studies and scientist's conclusions that saccharin is a "weak carcinogen", in particular acting in conjunction with other causes of cancer, such as cigarette smoking.



## FEATURES

# France plans new science museum

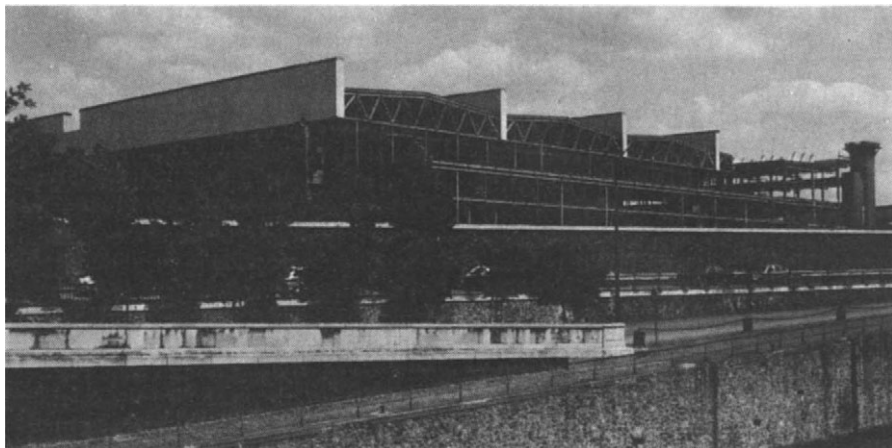
FRANCE is soon to publish a report proposing the construction of the largest science museum in the world. Compiled on the instructions of the President Giscard d'Estaing by Professor Maurice Lévy and a committee of 11, the report was completed last October after a 12-month study. However, Giscard d'Estaing has still not publicly released it; clearly there is a lively debate going on within the government about just how large a commitment France can afford to make to such a project at a time of economic squeeze. But there is no doubt that the project will go ahead in some form; even if money is hard to find, the advantages are too large to ignore.

First, there is the location. A disused abattoir called La Villette squats in the north-east corner of Paris. Its grounds cover 52 hectares, making it the largest open space in Paris outside the Bois de Boulogne. Abandoned almost ten years ago, the prey of property speculation and grandiose schemes, La Villette has long been the biggest property scandal in Paris. Thus its planned transformation into a park and a new Museum of Sciences and Industry (with a concert auditorium to be added later) will remove a painful reminder of a rather disreputable past. The conversion could be ready in five years at an estimated cost of 363 million FF.

Second, there is the equally scandalous condition of Paris's present science museum, the Palace of Discovery. Housed in a crumbling building constructed in the 19th century as a "temporary" exhibition hall, much of the equipment dates from the 1930s when Jean Perrin created it. Conceived of as one of a series of museums, it had to crowd in examples of all the sciences when the plans for the others were abandoned. Perrin's idea of "familiarising our visitors with the fundamental research by which science is created, through daily re-creations of the great experiments" has, through lack of funds and support, largely degenerated into formal lecture-demonstrations by underpaid staff to audiences increasingly made up of reluctant schoolchildren and middle-aged professionals. Meanwhile, the Conservatory of Arts and Crafts, set up at the time of the Revolution to be a collection of all the "originals of instruments or machines invented or perfected" in France, has remained a hodgepodge of machines, with almost no representation of very old, or very recent technologies, or those not originating in France.

So the decision to build a new museum of science, in La Villette, has a compelling logic.

Professor Lévy sees the museum as a counterweight to the way science is taught and popularised in France. As in most



*La Villette: a possible home for the new science museum.*

countries, the public view science as a formalised, completed structure, rigidly demarcated as the domain of "experts", pursuing their unfathomable goals by unknowable means. In France this is particularly marked, and leads to a virtual absence of public debate on scientific and technological questions. The new museum, Lévy feels, must be the initiator of such debates. "Science", he points out, "is too important to be left to the scientists."

Hitherto, most museums have followed one of three patterns. One such is typified by the Palace of Discovery: the hierarchal initiation of the layperson into the mysteries of science by the "expert", the quasi-academic lecture and demonstration. The conservatory of Arts and Crafts is an example of the second type: a dusty collection of artefacts, the frozen detritus of past accomplishments. Finally, there is the third kind, found in most postwar museums, where there is a tendency to represent science as a series of disjointed tricks, black boxes which, when their buttons are pushed by the visitor, dutifully trot out their magical effects.

But now modern technology and the increasingly sophisticated demands of the public, Lévy claims, has made possible "not an amelioration but a mutation" in the concept of a museum. New methods of communication will allow people to participate in scientific and technological decisions by giving them real access to the basic conceptual foundations of science and their practical consequences through active participation and debate.

This will happen in two ways. First, the flexibility of modern techniques permits "branching", the creation of an autonomous set of subthemes so that each museum visitor will be able to follow his or her own interests and needs in a certain subject, from basic concept to detailed application. Second, the museum at La Villette will make use of the "science in the streets" movement where scientists at

conferences also present their ideas in local public forums, answering questions and stimulating debate. La Villette plans to take this up in the form of a series of weekend events held every two or three months in which scientists and technologists would engage the public at open meetings at the museum as equal partners in a discussion of the consequences of what they have done.

There are a number of questions however which have been raised about the project. Bertrand Gille, an eminent French historian of technology, has pointed out that the relations of science to technology is far from unproblematic. A lack of historical perspective, the propagation of the idea that there is "one" technology flowing effortlessly from scientific advance are "received ideas" which still inform much thinking about the new museum, and tend to undermine the possibility of the critical approach that the museum is supposed encourage. Furthermore, Michel Biezunski, one of the originators of the interactive computer systems forming the base of the museum, is concerned about the control of the content of the displays. The public depends on the information in the exhibitions, and since both science and industry have a vested interest in stressing the positive side, where is the space for informed, critical debate?

Answering these criticisms, Lévy insists that the museum will try to show science as a "human endeavour", possibly by the inclusion of examples of scientific failures as well as successes. Further, he also insists that the museum staff, not government or industry, control the content of all exhibitions. But doubts persist. It is, after all, in the very nature of a museum to present historical successes, for they have become "knowledge". It may be felt that few people would come to see displays of technical failure, even though such exhibits might really illuminate how science works.

**Jim Ritter**



# UK underestimates cost of Trident deterrent

At present the British deterrent force consists of a fleet of four submarines armed with Polaris A-3 missiles that were supplied by the United States on terms well below cost in the 1960s, a largesse not likely to be repeated. The timing of the decision on renewing the deterrent is forced by the expected deterioration of the Polaris submarine hulls after 25 years of service, that is, by about 1993. Given the lead time needed for development and production of a new submarine, a decision is necessary soon. Postponement might mean abandoning the submarine-based deterrent, although choice of a different technology, such as air-launched cruise missiles, would imply a different timetable.

Coincidentally, NATO has just decided to push ahead with the modernisation of its 'theatre' nuclear weapons by deploying US Pershing II and Ground Launched Cruise Missiles (GLCMs); 160 of the latter will be based in England. Although in one sense these theatre missiles could be regarded as 'strategic' weapons — since like Polaris they will be able to reach targets within the Soviet Union — from an operational and financial perspective they are quite different. A new independent British deterrent force will be paid for by Britain, and, although it will be integrated into NATO forces, Britain will reserve the right to fire the missiles on its own. The theatre nuclear missiles will be assigned to NATO, but will be paid for by the United States and be under US control.

The decision to replace the Polaris fleet is first and foremost a political decision, based implicitly (for there has been no public discussion) on a certain view of Britain's status in the world and its relations with the US and its other NATO allies. The decision also has economic and technological consequences.

How much will a new submarine-based nuclear deterrent cost the British taxpayer? The operating costs of the Polaris fleet are not particularly large — only £126 million out of a total defence budget of £8,558 million in 1979 — but the investment in the design and production of a new submarine and missile will be heavy. Ian Smart in his 1977 Chatham House paper 'The Future of the British Nuclear Deterrent' estimated the cost of a replacement programme at £2,245-2,925 million at 1976 prices. That figure, swollen by inflation, stands now at

Britain has decided to renew its independent nuclear deterrent, probably by buying the submarine-launched Trident C-4 missile from the United States. **Judith Reppy**, Visiting Fellow at the Science Policy Research Unit at the University of Sussex, argues that this would be a questionable decision on economic grounds

perhaps £5,000 million for five submarines and their nuclear missiles.

There is reason, however, to question whether even this sum will pay the costs of replacing the Polaris fleet. The United States expects to spend over \$23,000 million (about £10,500 million) on a fleet of thirteen new submarines, and missiles; 62% of this is attributable to the cost of the submarine (see 'Trident — the US Experience: ADIU Report, Vol. 1 No 4, December 1979). The US Trident submarine is a huge boat, 560 feet long, and it has proved very expensive to build, costing over \$1600 million (£730 million) per boat. But even though Britain would surely choose a more modest design, it is still likely that it would cost more than Smart's estimate. That estimate allocated only 40% of the total programme costs to the submarine, or roughly £240 million per boat (1976 prices). The US figure suggests that is too low.

British shipbuilding capacity has shrunk since the Polaris submarines were

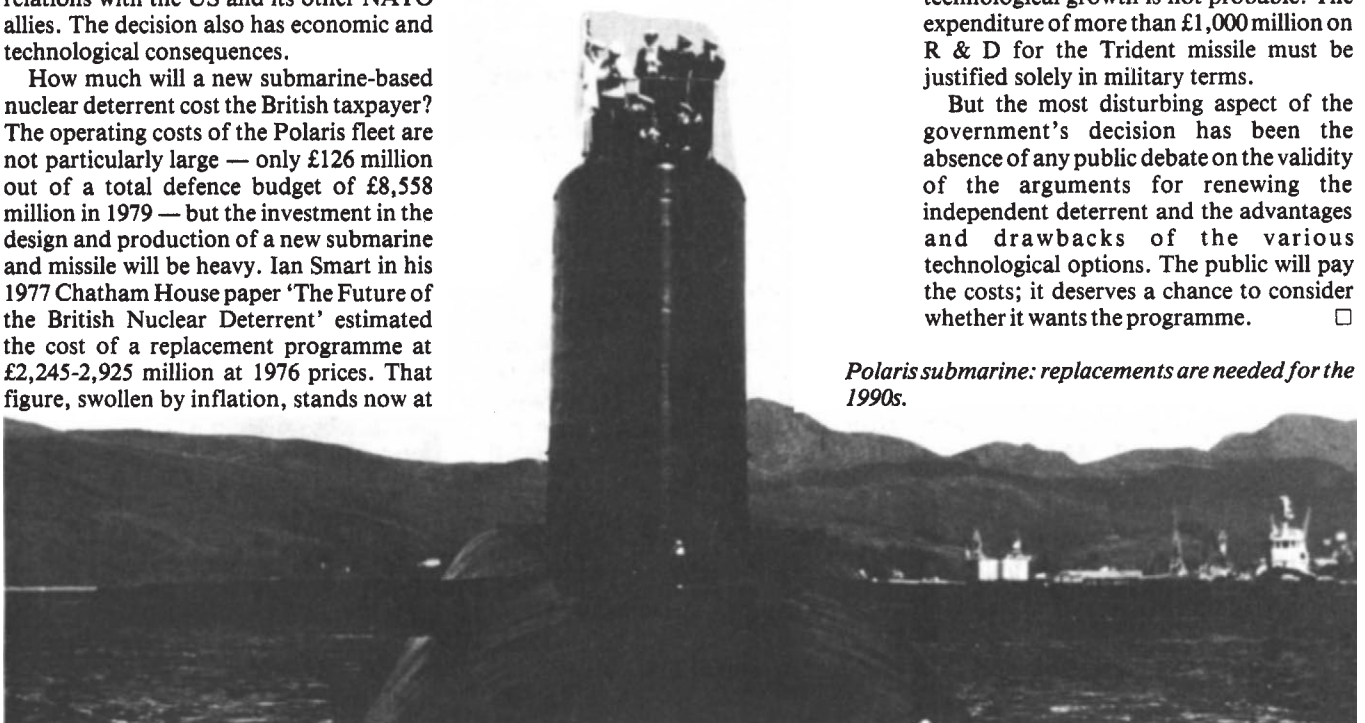
constructed in the 1960s, and only the Vickers Barrow yard now builds nuclear submarines. To build the new submarines at Barrow would take up capacity needed for other Navy programmes, whereas investing in new capacity elsewhere would entail costs not included in the Smart estimate. It can be argued that to invest in additional specialised industrial capacity to build five units of a product for which there is no other market would be economic folly.

Beyond these budgetary costs lie important opportunity costs, both with respect to other categories of public spending, such as education and health, and within the defence category itself. The share of the budget required for submarine design and construction will almost certainly pinch other elements of defence spending. Considering the emphasis placed on shortages of conventional weapons in the government's assessment of the military balance, it is perhaps surprising that tanks or air defence weapons are not considered a more urgent priority.

Given the large sums of money involved, the Trident decision also represents an important element in government funding for new technology. It is likely that the government will purchase the missiles from the United States, but develop and produce the warheads in Britain as was done for Polaris. Expertise in the technology of multiple independently targeted re-entry vehicles (MIRVs) will be acquired from the US. These capabilities, however, are not likely to spread into the civilian economy; they are too specialised for direct transfer, and the military market itself is so narrow that further internally-generated technological growth is not probable. The expenditure of more than £1,000 million on R & D for the Trident missile must be justified solely in military terms.

But the most disturbing aspect of the government's decision has been the absence of any public debate on the validity of the arguments for renewing the independent deterrent and the advantages and drawbacks of the various technological options. The public will pay the costs; it deserves a chance to consider whether it wants the programme. □

*Polaris submarine: replacements are needed for the 1990s.*



# Science in the 80s: the need for political involvement

**DD:** Ten years ago you wrote about the "party being over" for scientists. How do you feel that statement has worked out?

**SW:** Throughout the 1970s it has been more difficult for scientists to get money; to that extent my prediction was broadly right. In particular, the gradual reduction in per capita spending on the universities has meant that the British research councils have had to find a greater proportion of the total research expenditure than was the case in the golden years of the late 1950s and early 1960s. But I hadn't at the time thought that not only would the party be over, but that those who went to the party would be spending their time cleaning up afterwards — which seems to be the situation now. I hoped that we could get back to a steady, albeit modest, rate of growth, at least for the research councils; it's now clear that we will not see what I was hoping would be about a 3-4% real growth rate per annum over four to five years.

**DD:** What has been the main impact of economic stringencies in political terms?

**SW:** So far, the science budget has not tended to be very involved in either the politics of being cut or being expanded. It tends to stand slightly outside the rest of the political process, and therefore to run along with whatever is the overall tendency. In a period of expansion, the science budget tends to grow alongside; similarly it tends to contract during a period of contraction. And one of the things I was very anxious to do — and just succeeded in doing towards the end of my time as Secretary of State — was to cut the linkage between seeing the science budget as, in a sense, merely another expression of the "science and education" budget. I thought it had to be treated separately, because it had quite different cases to be made for it. I don't believe, for example, that the science budget is appropriately linked to the massive impact of demographic trends, on the education budget, whether rising as in the late 60s or now declining.

**DD:** What type of arguments did you use in Cabinet a year ago to obtain support for increased funding for research?

**SW:** I tried to explain in ways that would be understandable to the non-scientist — and to some extent I used myself as a litmus paper — what was going on in science, and what the prospects were for reasonable breakthroughs in some fields, or at least for some very fruitful work. Examples might be the work on laser technology being carried out by the Science Research Council, or the Medical Research Council's work in fields such as senile dementia and schizophrenia, research which offered some prospect of making

As Secretary of State for Education and Science in the last Labour Government, Mrs Shirley Williams (below) took an above average interest in the health of the scientific community. David Dickson talked recently to her at Harvard University, where she has just been a visiting fellow



substantial savings in the health service in the future, if one could find ways of treating people outside mental hospitals.

The idea was not necessarily to say "this is what the work being done is about" but rather "these are examples which readily lend themselves to being thought about in terms of their impact on other people's programmes". I didn't feel there had been a sufficient attempt to explain the research council's activities, as distinct from saying "here's the science budget; I want a 2% growth for it". In other words, there had not really been an argument about the priorities for scientific research as against the priorities for other things, such as defence or social security.

My own belief is that, although scientists don't like to think that research is involved in the political process, it has to be, at least to the extent that science is explained as proselytised about. It wasn't really necessary at the time of the great Robbins expansion of higher education in the 1960s, because the dual support system bore the expansion on its back. Now that system is threatened by demographic shifts, as well as the general cutting back of the higher education budget — which makes it important for scientists and the research councils to learn to put across what they are doing, and build up a lobby of support.

**DD:** Turning to the social aspects of scientific growth, how do you feel the relationship between the scientific

community and the political community has changed in the 70s?

**SW:** In the narrow sense of the two communities, I would firstly sum it up by saying that the scientific community resisted getting involved, or even communicating much, with the political community, and it certainly seemed to me that such communication was very important. The process under which, for example, the science and technology committee of the House of Commons has tended to cross-question civil servants, and even to some extent the heads of research councils — even though the witnesses sometimes feel they are not revealing vast knowledge — will only work effectively if scientists are willing to make themselves available to the political community. I would criticise scientists in Britain by saying that, in general, they have not been all that readily available, and have been a bit suspicious of people taking time off from the lab to talk to "those amateurs out there". I think that's rather a mistake.

**DD:** You have frequently suggested that there should be more of a scientific lobby. Do you feel there has been any movement in this direction?

**SW:** A little. The research councils, and particularly the heads of the research councils, have done much more to come out and explain what they are doing. And insofar as the barriers between the sciences are beginning to break down — for example through joint research projects between the different councils — as well as to some extent the barriers between the scientists and the press, then the research councils are pushing in the right direction.

**DD:** But once money begins to get tight, are you not required to make a better case justifying support?

**SW:** Yes, and I think that's right. One parallel that has always struck me forcefully is that you could increase the arts budget by 15%, and the entire arts lobby would shout its head off about how mean it was, because they always want at least 25% increase a year. But if you increase the science budget by 5% in real terms — say 11 or 12% in money terms — scientists would always be rather grateful. Now I think that the arts lobby overdid it, but they have a large lobby of people who will write letters to the newspapers, speak in the House of Lords, and so on. The scientists — who, thank goodness, are at long last beginning to appear in the Houses of Parliament — are not so ready, don't react so quickly, don't fight so hard. I wouldn't want to push it so far as the arts lobby does, which may even be slightly counterproductive, but I still think there is something to be said for closing the gap a bit.



**DD:** How do you feel about the way that concerns about the social impacts of science have been institutionalised — the Genetic Manipulation Advisory Group is the obvious example — and of the way that the scientific community has responded.

**SW:** There have been many types of responses. GMAG is only one example, and indeed the fact that the Association of Scientific, Technical and Managerial Staffs recently held a conference on GMAG is a good example of how questions about the social impacts of science have been spread beyond the small community of scientists and administrators that directly interweave with them. The Windscale Inquiry is another good example, which was rather well handled, if one compares it with some of the tremendous clashes that there have been in France and Germany. The whole idea of having an inquiry of that kind, with a judge in charge, but passing the conclusions on to the House of Commons for a free vote, was rather an innovative way of going about an area where science and social policy come up against one another.

One example where this went badly was in the area of dangerous pathogens — the smallpox case at the University of Birmingham; and that was because the dangerous pathogens' rules and regulations pre-date public interest in the field of science and social policy, so that what we had was a relatively secretive and relatively unpublicised system of control and regulation. Immediately something went wrong, the Shooter Report on the smallpox case was able to pinpoint all sorts of holes in the system, because it hadn't really been looked at. People had become complacent about it; it wasn't within the light of the public domain. GMAG was; nuclear power to some extent is, although I would like to see a broader range of public interest brought in; and this is also particularly true in the area of advanced medical science.

As for GMAG, as a device for meeting public concern and, in the initial stages, for allowing experiments to be conducted in closely controlled situations, it had a great deal to be said for it. As a step into a whole new area of science with a great number of public fears associated with it, you only needed to have one accident for the whole of genetic engineering to be at great risk, and for some parliamentarians to say "ban the lot". Indeed there was quite a strong move in Parliament to say that we should not do some of the experiments that fell into the containment categories. And so although I know now that some scientists, and even more industrialists, would criticise GMAG as being exceptionally restrictive, nevertheless as a way of showing that public fears were being taken seriously it was an important beginning. The risks may be low; but what's at stake is so high that the risk-benefit equation does involve a great deal.

What bothers me about the US is that the controls and regulations, such as they have

been — I use that tense advisedly — do not really extend very much into the private sector. And, of course, there are countries, such as Switzerland, where the private sector is relatively unregulated. Given that what we are looking at should be an international system of regulation — you can erect national systems, but what difference does that make to a virus? — one has to make some sort of a compromise. I realise that it would almost certainly not be as rigorous as the current GMAG rules to be widely acceptable, but one hopes it would have rather more sensible safeguards, especially with regards to category III and category IV experiments. Britain might have a more regulated national system than some other countries; but the general principles of good practice at the bottom end and pretty strict regulation at the top end is something we ought to try to get across through the European Science Foundation, and in other ways.

**DD:** The US is currently experiencing a political reaction against regulation, and is caught in the trap while move is being learnt about potential environmental and health hazards, the less regulation becomes politically acceptable. How do you correlate social and political needs?

**SW:** The way I see it, the US often needs regulation to help make sense of its legislation, which has often been cast in the form that will generate sufficient support — legislation emerging from a process of creating a coalition to get it through. Regulation then becomes the way that you rationalise this rather strange cross-path of legislation. If one takes the fields of education or employment — or for that matter safety and health — one finds that the regulations are extremely detailed and precise, but that they are often changed and amended in the light of events. So that you find people responsible for regulation almost going round the bend with the sheer weight, change and detail of the regulation. And that, in turn, leads to the kind of antithesis of people like Senator Edward Kennedy, for all that he is a liberal senator, moving against regulation.

**DD:** How do you see the future prospects for science in Britain? What are the new political pressures likely to be in the 1980s?

**SW:** One important need is for the leaders of the scientific community to get across the idea of lead-times; in other words, to try to get agreement as far as possible that a substantial chunk of the budget ought, as far as possible, to be insulated from year-to-year changes. I'd like to see the budget split between extra money that could be used quickly, and money which requires to be committed for some time, since one of the most difficult things to do is to set up a team of people and then to discover a year or two later that the team is chopped off from its funds, or from half of its funds, just when it is getting going.

Secondly it is important to move in the

direction that Geoffrey Allen of the SRC talks about, namely providing fellowships on a four- or five-year basis for young scientists. I am very concerned that the profile of the academic world is such that unless we are very careful, some of the most able of the new generation of young scientists will not get jobs in universities.

**DD:** But looking at the question the other way round, what new challenges can you see science having to face from the political body — or from the social body — to which it will have to respond?

**SW:** One area in which a great deal of thought is needed is the extent to which the 'sophistication factor' is likely to have an impact on certain major areas of public finance, particularly in health. A scientist once said to me that if heart transplants had been generally successful, the National Health Service would be in even more trouble than it is, and that seems to me correct. We should be worrying whether the sophistication factor, particularly in medicine, will in practice mean that one is budgeting against the needs of the great majority of the population. It is extremely important to notice how anti-distributive the effects of advanced medical technology can be. Look at the US where medicine has become so expensive because of the technology that, in practice reasonably decent intermediate technology medicine is out of the range of part of the population.

I believe that the government — particularly people concerned with the health field, though pensions and mental health also come into this — should decide, together with scientists, what profile of research is likely to ease the position of hard-pressed health budgets, and what research is likely to make it worse.

People should be aware of the costs as well as the benefits of successful research. Even in the US, in the discussions that I have followed here on medical insurance schemes, almost nobody has taken up the theme of the massive consequences of rapidly advancing medical technology in terms of health care. If I can be blunt, I think that in ten years time it will be impossible to finance any effective public medical service in the US, because a great range of less expensive but reasonably effective technologies will have been ruled out.

**DD:** Does this mean you see the science budget more and more closely linked to other areas of government spending and social policy? How can the social impact issues be brought into the policy process?

**SW:** I think that it has to be done at every level. Certainly the research councils should take such matters into consideration. But one would also have to have a kind of parallel system, parallel meetings of interest groups in the House of Commons and in the mass media. This would mean the scientific community being brought together with economists and social scientists more often to look at the implications of certain advances. □

# CORRESPONDENCE

## Indian science fails to meet basic needs of the country

SIR, — Anil Agarwal's article on 'Indian Science' brought out several interesting points. However, he has failed to raise some basic issues. For example, while India is spending money for highly sophisticated technology such as space and nuclear research, science and technology, even in their crudest form, have not reached the mass of Indian villagers, for whom hunger and starvation are an everyday reality. Indian science policy was not able to prevent the Bihar famine and recent floods and cyclone disasters. It has, however, led to the creation of bureaucratic research institutions and to the mushroom growth of CSIR research laboratories. It is, in fact, only the continuation of a policy which existed in colonial times. Research institutions such as CSIR, the Atomic Energy Commission and the Defence Research Laboratories are prestige institutions. Most Government bodies are bureaucratic institutions controlled by a few individuals.

India has built up a sizeable infrastructure for research and industry, but technology for manufacturing common household goods, such as dry batteries or condensed milk, still has to be imported. Indian scientists have given no thought to the need of basic technology in their country. Despite the green revolution, agricultural wages have declined or stagnated in real terms. The population of cities is increasing annually by about 5% because of the influx of the impoverished from the countryside.

The present attempts to formulate a science policy do not account well enough for education. The type of science education given in Indian universities is imported from western countries, and has no relevance to Indian society. A large amount of money has been spent on universities, research institutions and engineering colleges. At the moment there are more than 3,000,000 students in universities, more than one third of them being science students. By the end of 1979 the figure will rise to more than 5,000,000.

Indian universities have become factories for producing graduates. But why expand like this when there are not enough jobs for those who already have degrees? Recently there were more than 40,000 applicants for 40 vacancies; and for a few vacancies in nationalised banks there were so many applications that they had to be collected in vanloads. Unemployment among graduates runs at more than 30% and in large cities the situation is really alarming. Even though most universities provide undergraduate science and technology courses, the standard is pretty bad. That of science teaching in private colleges (which are affiliated to the universities but run on a commercial basis) is far below normal. Most teachers do not have sufficient qualifications to teach at this level and laboratory facilities are very bad.

What is the point of producing graduates who do not come up to reasonable standards and who do not serve any useful purpose in Indian society? Instead, science education should aim to teach basic ideas on a *mass scale*. The standard of science teaching in schools must be improved before talking about undergraduate study. Most schools in small towns have to be content with spirit lamps, and experiments are not devised to make maximum use of existing facilities.

It would, for example, be a good idea to use experimental kits (like those devised by the Open University) which could be stored in small towns, each store forming the nucleus of

a science centre. Mobile service units, manned by trained teachers and operating from those centres, could go to villages and explain to children, with the help of simple experiments, the concepts of basic science and technology. So far Indian science education has no relevance to Indian society.

Although a lot of noise has been made in favour of appropriate technology, it has failed to make any impact on rural development in India. This is because appropriate technology by definition is an indigenous self-generating process arising as a response to the economic and social needs of various forms and levels of society instigated and initiated by the users of existing technology. Such technologies will not work satisfactorily until farmers have experienced the benefits of communal production and projects and are thus able to design and operate such equipment. Unless the desire for change and for an appreciably higher standard of living takes place in the peasant communities (which is happening among Punjab and Haryana farmers) new techniques will not be accepted or exploited fully.

At present the keystone of Indian science education is elitism and personal achievement rather than social responsibility; centralisation rather than dispersion; urban rather than rural development; consumption rather than aesthetic development. What is important and essential is that basic science and technology should be oriented towards the needs of the Indian society. Above all, the masses must be carried with technological development and not isolated from it.

Yours faithfully,

A. VAIDYANATHAN

The Open University, Milton Keynes, UK

## Nuclear secrets

SIR, — We concur with the sentiment expressed in your editorial *Nuclear secrets: no clear frontiers* (18 October page 511) that the "narrower question of American security should yield to the broader one of global non-proliferation". But we hope you did not mean that articles about hydrogen bombs should not be published simply because of "sincerely held views" that such articles contribute to proliferation.

Neither of the disputed documents could be of aid to a state in the initial acquisition of nuclear weapons, because the documents discuss thermonuclear weapons, for which a fission weapon capability is a prerequisite. Moreover, most of the dialogue and court proceedings was in the broader context of global non-proliferation.

Months ago, before the temporary restraining order was issued by Judge Warren, we technically reviewed Morland's article for *The Progressive*. The article and letter do not contain design details; rather they discuss various — oftentimes incorrectly applied — concepts already known to scientists throughout the world. It is therefore difficult to understand how such an article can further affect the already precarious international situation we now face.

Technical information is essential to informed political debate on an enormous range of issues. For example, thinking only of current policy debate concerning nuclear weapons and nuclear arms limitation, we might list the following: the SALT treaty, the necessity of the MX missile, its siting alternatives, and a comprehensive nuclear test ban.

The US and other nuclear weapons-states must face the fact that five nations, without help, have successfully undertaken the industrial and scientific effort necessary to

make hydrogen bombs. The average length of time to achieve a fusion explosion has been five years after first testing a fission device. That this average was as long as five years is more likely due to political indecision and industrial development time rather than a lack of understanding of elementary concepts.

Motivation and resources are far more important determinants than underlying concepts. It is still prudent for governments to avoid authenticating (through their powers of classification) partially correct public information, such as the Morland article and various letters. But mainly they should concentrate on protecting their own ultra-sensitive official documents containing weapons-design data, for example UCRL-4725 and -5280, which the US Government erred in making available to the public.

The issues involved in the case go far beyond the question of weapons data alone. For example, the US Government in its brief to the 7th Circuit Court of Appeals in Chicago raised the argument that "technical data" are not constitutionally protected under the First Amendment because they are not an "essential part of any exposition of ideas" and are not of any "social value as a step to truth". In a world as technologically oriented as ours, such an argument is not only nonsensical and false, it is dangerous to the functioning of free societies.

One of the issues raised by Morland is that unnecessary secrecy can tranquillize public debate. Partly because of reliance on technology-denial and secrecy, several US Administrations have avoided a moratorium on testing, production, or deployment of nuclear weapons — although such restraint would retard proliferation far more substantially than does the illusion of security through technology-denial and secrecy. The most urgent need is a universal nuclear test ban, which might be introduced as an amendment to the Nuclear Non-Proliferation Treaty.

All of the signatories to this letter are engineering physicists who have filed affidavits regarding the various letters and articles relevant to *The Progressive* case and two of us have had access to the classified and "in camera" documents. This letter has no official connection with the Argonne National Laboratory, with the University of Chicago, nor with the UK Department of Energy.

Your faithfully,

GERALD E. MARSH

ALEXANDER DE VOLPI

GEORGE S. STANFORD

Argonne National Laboratory,  
Argonne, Illinois

## Exposing sociobiology?

SIR, — I am pleased to detect a slight shift in *Nature's* editorial predilections, from totalitarian socialism to totalitarian Mahometism. At least, as Mr Crouchback would say, they have a king and some sort of religion.

Perhaps you will now find it possible to expose the sociobiological theories as no mere appendage of Naziism, but the very emanation of Satan; whose hand can now be discerned — in the horrific decline in spirituality — to say nothing of the savage cuts in public expenditure, and insecurity of tenure in medical research jobs — that has plagued the West since the Turk was stopped at the gates of Vienna.

Your faithfully,

CHARLES W. CLARK

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## NEWS AND VIEWS

## A new twist for DNA?

from David R. Davies and Steven Zimmerman

In the twenty-seven years since Watson and Crick proposed that B-DNA is a right-handed double helix, the concept of a regular DNA structure, varying only in the chemical sequence of its bases, has become one of the foundations of molecular biology. Although fibre diffraction of DNA shows the existence of at least three distinct forms, thereby demonstrating the flexible nature of the sugar phosphate backbone, the structure in solution has always been believed to be a regular B form. Occasionally, suggestions have been made that some DNAs, particularly with unusual base sequences, could adopt significantly different conformations, but the evidence has never been conclusive.

This situation has recently changed dramatically. There are now two clear examples, both deoxypolymers with simple alternating sequences, for which strong evidence exists that the conformation of the backbone in solution is also alternating. In one case, poly(dA-dT)·poly(dA-dT), the current model is still a right-handed double helix similar to B-DNA in general form. For poly(dG-dC)·poly(dG-dC), on the other hand, a remarkable left-handed structure has been proposed by Wang *et al.* (*Nature*, **282**, 680; 1979) on the basis of the elucidation of the crystal structure of the hexanucleotide d(C-G)<sub>3</sub>. In this crystal two molecules are combined with Watson-Crick base pairing to form a double helical segment with remarkable internal regularity. In addition, neighbouring segments pack on top of one another to approximate an infinite helix with antiparallel strands and twelve base pairs per turn. This structure has been termed 'Z-DNA'.

In this new Z helix the conformations of the guanine and cytosine deoxynucleotides differ considerably from one another so that the repeating unit becomes a dinucleotide, unlike the B-DNA helix in which the repeating unit is a single nucleotide. The torsion angles of the bases relative to the sugars are quite different. Whereas the cytosine adopts the *anti* conformation relative to its deoxyribose as

in B-DNA, the guanine adopts the *syn* conformation. The puckers of the two deoxyribose rings are also different, being C3' endo for two internal deoxyguanosines and C2' endo for the deoxycytidines. Finally, the conformation of the backbone about the C4'-C5' bond is *gauche-trans* for the dG residues and *gauche-gauche* for dC. The net result of these differences between the purine and pyrimidine nucleotide conformations is to produce a staggered zig-zag course for the deoxyribose phosphate backbone, hence 'Z-DNA'.

These single crystal results imply strongly that this hexanucleotide structure could exist in a polymer of deoxynucleotides. Indeed, Arnott *et al.* (*Nature* in the press) have now observed a form of poly(dG-dC)·poly(dG-dC) that they believe from X-ray fibre analysis to be the Z-form. The helical parameters of this fibre pattern show a remarkable correspondence to those of the crystal. The former have a helical pitch of 43.5 Å with 12 base pairs, compared with the prediction from the crystal of 44.6 Å. The fibre also provides evidence for a dinucleotide repeating unit.

Arnott *et al.* (*op. cit.*) have observed that the same fibres that initially gave B-form diffraction patterns later gave Z-form patterns. This implies that the molecules can undergo a change of direction of the helix twist within the fibre in a relatively confined space without greatly altering the orientation of the double helices and without getting significantly tangled. Wang *et al.* (*op. cit.*) point out that the transformation between Z and B will involve separation of the base pairs, a large rotation of the guanine base accompanied by a rotation of the entire deoxycytidine residue, followed by rejoining of the base pairs. They note that the junction between Z and B-DNA will have a stacking discontinuity which might result in a kink in the molecule.

There is evidence that the Z-DNA conformation is not restricted to poly(dG-dC)·poly(dG-dC). Arnott *et al.* report the observation of this form in fibres of poly(dG-dT)·poly(dA-dC). They also interpret the X-ray diffraction results of Saenger *et al.* (*Jerusalem Symposium on Quantum Chemistry and Biochemistry* V, 457; 1973) for the alternating copolymer of deoxyadenylate and 4-thiothymidylate in

terms of a modified Z helix with 14 base pairs in a pitch of 54 Å.

The anomalous behaviour of poly(dG-dC)·poly(dG-dC) has been known for some time. Pohl and Jovin (*J. molec. Biol.* **67**, 375; 1972) were the first to observe two distinct forms of poly(dG-dC)·poly(dG-dC) in solution, with a cooperative transition occurring between them at high salt concentrations. The circular dichroism spectrum of the high salt form is nearly an inversion of that of the low salt form, which in turn resembles that of B-DNA. This suggests that the transition to the high salt form involves a rearrangement of the bases. Wang *et al.* (*op. cit.*) believe that their Z-form corresponds to the high salt form, even though the d(C-G)<sub>3</sub> was crystallised at relatively low salt concentrations. This interpretation is consistent with the results of a previous NMR investigation of (dG-dC)<sub>8</sub> by Patel *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **74**, 2508; 1979), who clearly demonstrated that the repeating unit in the high salt structure is a dinucleotide, and that marked differences between the two phosphodiester linkages (that is, dCpG and dGpC) give rise to two peaks in the high salt <sup>31</sup>P NMR spectrum. Differences in the sugar hydrogen resonances of the two forms also led to a prediction of a dramatic change in either the guanosine or cytosine glycosidic torsion angle during the transition from the low to high salt form.

How do these results relate to the 'alternating B-DNA' model recently proposed for poly(dA-dT)·poly(dA-dT), (Klug *et al.* *J. molec. Biol.* **131**, 669; 1979)? This model is based on the crystal structure of the tetranucleotide d(A-T)<sub>2</sub> (Viswamitra *et al.* *Nature* **273**, 687; 1978) which also has different nucleotide conformations, with the thymidine sugars in the C2' endo conformation while the deoxyadenosine sugars are C3' endo; in addition the O-P torsion angles are *gauche gauche* for the dA(3'5')dT segments and *trans gauche* for dT(3'5')dA. Klug *et al.* used this result as the basis for a hypothetical 'alternating B-DNA' structure for polynucleotide sequences containing alternating purines and pyrimidines. Although it has an alternating backbone, this structure is closely related to B-DNA and is quite different from Z-DNA. The NMR analysis of fragments

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of poly(dA-dT)·poly(dA-dT) 145 base pairs long supports the concept of an alternating backbone conformation with a dinucleotide repeat, the  $^{31}\text{P}$  resonance showing a small splitting (Shindo *et al.* *J. biol. Chem.* **254**, 8125; 1979). Also, in a joint X-ray/NMR study on fibres of poly(dA-dT)·poly(dA-dT), (Shindo & Zimmerman, *Nature* in the press) calculated NMR resonances that are consistent with the 'alternating B-DNA' model of Klug *et al.* As the 'alternating B-DNA' structure was also anticipated to occur for poly(dG-dC)·poly(dG-dC), it is surprising that the low salt form of this polymer shows no splitting of the  $^{31}\text{P}$  resonance.

We should point out that the various models that have been proposed for left-handed or side-by-side DNA of irregular sequence (references may be found in Crick *et al.* *J. molec. Biol.* **129**, 449; 1979; *News & Views* **278**, 780; 1979) are quite different from Z-DNA and from the alternating B-DNA structure, both of which seem to be a response to a regular purine-pyrimidine alternating sequence.

Is the left-handed Z-form likely to be found in DNA *in vivo*? Where there are stretches of alternating GC sequence (or possibly even some other kinds of alternating purine-pyrimidine sequences), the potential will exist for forming this structure. However, the transition from B

to Z occurs at the rather unphysiological salt concentrations of 2.5 M NaCl or 0.7 M  $\text{MgCl}_2$  (Pohl & Jovin *op. cit.*) which might seem to make the Z-form unlikely except in halophiles; nevertheless, complex ions such as polyamines or specific binding proteins might favour the transition. It is interesting that the association of complementary closed circular single-stranded DNA molecules can occur over as much as 70% of the length of each DNA strand (Stettler *et al.* *J. molec. Biol.* **131**, 21; 1979). Topological considerations and the observed circular dichroism changes suggest that some segments of this DNA are left-handed but the extent, regularity and conformation of these segments are not known. If left-handed helical regions do occur in DNA *in vivo*, they might be associated with significant biological effects through their interactions with DNA-specific enzymes and binding proteins. Also, the presence of the Z-conformation in supercoiled DNA would influence the interpretations of supercoiling. It has been suggested by Gellert and Mizuuchi that the energy stored in supercoiled DNA might cause appropriate alternating sequences to snap over into the Z form even at physiological salt concentrations. On the basis of the detailed structural information now becoming available, experiments can be designed to test these possibilities. □

## Quick-freezing — the new frontier in freeze-fracture

from David Shotton

A TECHNIQUE for the ultrastructural preservation (fixation) of biological specimens by extremely rapid freezing in the absence of cryoprotectants has been developed for freeze-fracture and thin-section electron microscopy by John Heuser (of the Department of Physiology at the University of California Medical School in San Francisco) and Thomas Reese (of the National Institute of Neurological and Communicative Disorders and Stroke, at the National Institutes of Health in Bethesda). This promises to open a new chapter in biological ultrastructure research, because it now makes possible electron microscopic studies of biological events which occur within the millisecond time range, permits the observation after freeze-etching of surface features previously obscured by non-volatile chemical cryoprotectants, and allows a critical evaluation to be made of the artefacts produced in biological specimens by the chemical fixation and cryoprotection techniques routinely used by electron microscopists.

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Results which Heuser, Reese and their colleagues have obtained by using the quick-freezing technique on three distinct biological systems have recently been published in three beautifully-illustrated papers contained in the latest volumes of the *Journal of Cell Biology* (Heuser *et al.* *J. Cell Biol.* **81**, 275; 1979; Heuser & Salpeter *J. Cell Biol.* **82**, 150; 1979; Chandler & Heuser *J. Cell Biol.* **83**, 91; 1979), giving us a chance to assess the significance of this development.

In essence the technique is very simple, and involves bringing a biological specimen, fixed beneath the lower end of a vertical falling rod, rapidly into firm contact with the upper polished surface of a pure copper block cooled from below to approximately 4 K ( $-269^\circ\text{C}$ ) with liquid helium. Their first quick-freezing machine, described by Heuser, Reese and Landis in 1976 (*Cold Spring Harbor Symp. quant. Biol.* **40**, 17), was developed from the design originally put forward in 1964 by Van Harreveld and Crowell (*Anat. Rec.* **149**, 381; Van Harreveld *et al.* *J. Cell Biol.* **25**, 117; 1965). It resembled in principle the simple liquid nitrogen-cooled copper block

freezing method of Dempsey and Bullivant (*J. Microscopy* **106**, 251; 1976), although it had the important advantages of automation, a colder initial block temperature and a sophisticated specimen mounting system which minimised contact damage.

An improved version of their apparatus is now described by Heuser *et al.* (*op. cit.* 1979). With it they could freeze the surface 10  $\mu\text{m}$  layer of living specimens within 2 ms, with excellent ultrastructural preservation and minimal ice crystal damage. Further from the surface, the inherently low thermal conductivity of biological tissue limited the rate of heat loss and unavoidably led to the growth of large ice crystals and consequent tissue damage, similar to that observed whenever tissue is frozen without previous chemical cryoprotection by conventional techniques of immersion into freezing Freon-22 or nitrogen slush.

Once frozen by the quick-freeze method, specimens can either be freeze-fractured and replicated in the normal way, care being taken to ensure that the fracture plane passes through the surface 10  $\mu\text{m}$  layer, or alternatively can be freeze-substituted and chemically fixed at  $-95^\circ\text{C}$  in a thawing acetone-osmium tetroxide mixture before embedding for thin-section electron microscopy. One particular advantage of this copper block quick-freezing method over liquid propane jet-freezing methods, which also give improved rates of cooling, is that the well-frozen layer of the specimen is physically flat and parallel to the specimen support, enabling extensive freeze-fracture replicas from this region to be obtained using a Balzers microtome system.

The most important and exciting advantage of this quick-freezing method, in comparison with conventional ultrastructural approaches, is that it enables living specimens to be instantaneously frozen with good ultrastructural preservation at known intervals after a biological stimulus, and so makes it possible to use the electron microscope for kinetic studies of transient biological events which are completed within a few seconds or even, with certain favourable specimens, within a few milliseconds. It was for this purpose — to visualise the train of events which occurs at the presynaptic membrane of the frog neuromuscular junction during the first few milliseconds after the arrival of a nerve impulse — that Heuser and Reese originally developed their apparatus. The results they obtained are presented in the papers by Heuser *et al.* (1976 and 1979, *op. cit.*) and Heuser *Soc. Neurosci. Symp.* **2**, 215; 1977), and a further paper now in press.

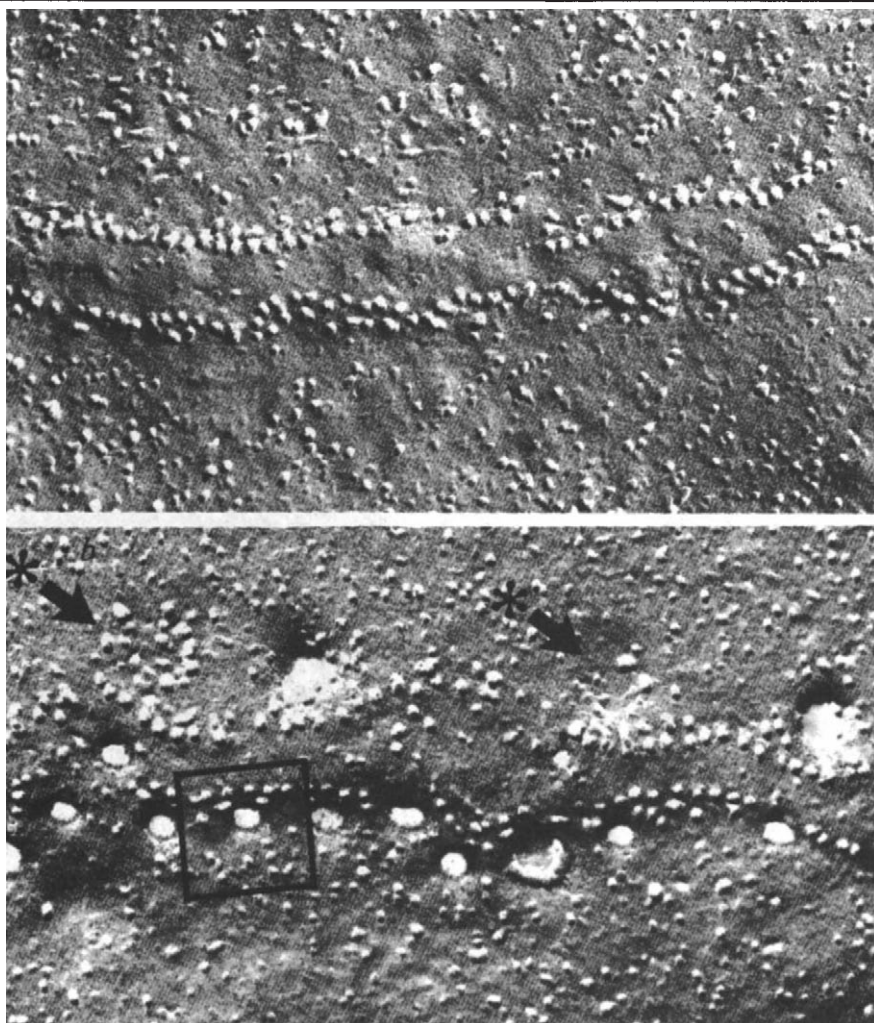
Living cutaneous pectoris muscles were dissected intact from frogs, with their motor nerve attached. Each thin sheet-like muscle was individually mounted onto the specimen support of the quick-freezing machine, and was caused to contract by



electrical stimulation of the nerve as it fell towards the cold copper block. The interval between the arrival of the nerve impulse at the neuromuscular junction and the moment at which the specimen froze solid was recorded on an oscilloscope, and was later related to the appearance of the presynaptic membrane after that specimen had been freeze-fractured, enabling the investigators to determine the time course of the exocytotic events caused by the fusion of the synaptic vesicles with the presynaptic membrane. 4-aminopyridine (4-AP) was used to increase the number of acetylcholine neurotransmitter quanta discharged with the arrival of each nerve impulse from the normal level of less than one quantum per presynaptic active zone of transmitter release to a level at which they could more easily be studied. No structural changes were observed during the first 3 ms after stimulation (Fig. 1a), but by 5 ms narrow exocytotic openings were visible at fusion sites all along the active zones (Fig. 1b). During the subsequent 20 ms, these fused vesicle membranes collapsed and flattened out, leaving only faint depressions in the postsynaptic membrane, each containing two or more particularly large intramembrane particles not previously present on the presynaptic membrane. The subsequent fate of the vesicle membrane components thus inserted into the presynaptic membrane was followed by quantifying the movement of these large intramembrane particles over the next 400 ms, during which period they moved away from the active zones towards sites of coated vesicle endocytosis, in the region between adjacent active zones.

The ability to undertake such direct kinetic studies represents a significant breakthrough in cell biology, since in the past kinetic sequences of rapid events at the ultrastructural level could only be deduced from static electron micrographs of chemically-fixed specimens, arranged into postulated temporal sequences on the basis of the investigator's preconceptions of what might reasonably be expected to occur. For neurobiology in particular, these studies provide further strong direct support for the synaptic vesicle fusion and recycling scheme first proposed by Heuser and Reese in 1973 (*J. Cell Biol.* 57, 315).

The second advantage of quick-freezing is that, by being able to freeze delicate biological specimens from dilute aqueous solutions in the absence of chemical cryoprotectants without ice crystal damage, one can then expose the outer surfaces of membrane, extracellular structures and intracellular cytoskeletal elements by freeze-etching, a technique in which water molecules are allowed to sublime from the frozen surface of the fractured specimen before replication. In the past this technique could only be applied with any degree of success to rather sturdy small cells in suspension, such as erythrocytes, yeast and bacteria, which could withstand conventional freezing



**Fig. 1** *a*, High magnification view of the 'P face' of one active zone from a frog motor nerve terminal soaked in 1 mM 4-AP and 10 mM  $\text{Ca}^{2+}$  for 30 min and given one stimulus 3 ms before freezing. By 3 ms, the action potential presumably arrived in the terminal, but it did not alter the resting appearance of the active zone, which is recognised as a slight ridge bordered by parallel rows of large intramembrane particles.  $\times 143,000$ . *b*, P-face view of an active zone from a nerve terminal treated like the one in *1a*, but given one nerve stimulus 5 ms before freezing. In the interval between 3 and 5 ms, just at the time when the nerve begins to discharge large numbers of acetylcholine quanta, many membrane perturbations appear along the edges of the active zone. These are believed to be exocytotic stomata, or openings into the underlying synaptic vesicles. The two areas marked by asterisks are thought to be vesicles that have collapsed flat after opening; they display a telltale cluster of two or more large intramembrane particles like those found in intact synaptic vesicle membranes. The square encloses an early stage in one characteristic exocytosis event.

procedures without cryoprotection.

Heuser and Salpeter (1979 *op. cit.*) have studied unprotected quick-frozen postsynaptic membranes from the electric organs of the electric rays *Torpedo californica* and *Narcine brogiiensis* in this way. Besides giving us fascinating views of the structure of the basal lamina within the synaptic cleft, of intracellular cytoskeletal filaments and of synaptic vesicles, they have revealed that the acetylcholine receptor molecules form striking extensive ordered arrays, which can be seen not only as protrusions on the etched external surface of the postsynaptic membrane (Fig. 2a), resembling those previously reported on etched *Torpedo* membrane vesicles by Cartaud and his colleagues (*FEBS Lett.* 33, 109; 1973; *Proc. 6th European Congress on Electron Microscopy, Jerusalem*, 336; 1976; *J. Cell Sci.* 29, 313; 1978) but also as low-profile

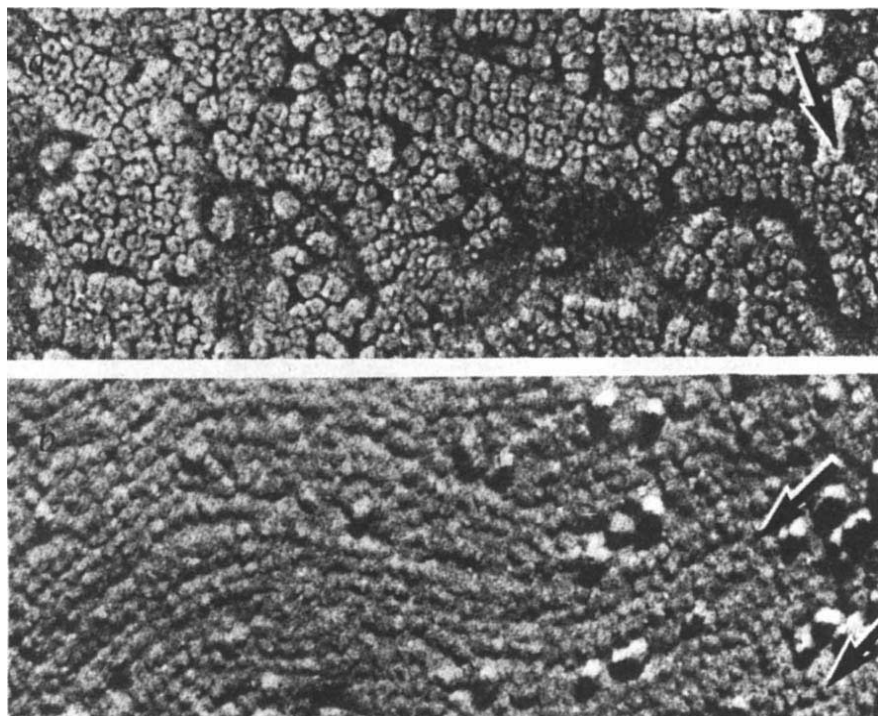
particles on the E fracture face of these unfixed quick-frozen membranes (Fig. 2b). Orci, Perelet and Dunant (*Proc. natn. Acad. Sci. U.S.A.* 71, 307; 1974) had previously obtained limited views of such E-face particle arrays, but in others' hands they have usually been rendered invisible by artefactual changes resulting from glutaraldehyde fixation and glycerination. They occur at a density ( $>10,000 \mu\text{m}^{-2}$ ) which correlates well with previous physiological and autoradiographic estimates of the receptor packing density. This density is much higher than that of the larger intramembrane particles seen on the fracture faces of both fixed and unfixed membranes, which had often previously been assumed to represent the receptor molecules.

The third advantage of the quick-freezing method is that it enables one to evaluate the artefacts produced in



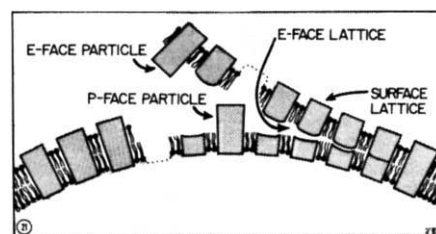
biological specimens by the alternative chemical fixation and cryoprotection techniques normally used by electron microscopists. One example of this, from the work of Heuser and Salpeter, is the striking differences in appearance and aggregation state of the intramembrane particles on the P and E fracture faces of *Torpedo* postsynaptic membranes when freeze-fractured after quick-freezing and after glutaraldehyde fixation, glycerination and Freon freezing. Other examples of such artefacts, which are important in assessing current theories of membrane fusion, come from the studies which Chandler and Heuser (*op. cit.* 1979) have made of cortical granule exocytosis in fertilised sea urchin eggs. In this work, the authors used the quick-freeze technique to capture stages in the 'cortical reaction' which originates from the point of entry of the sperm and which within 30 s sweeps across the entire surface of the egg. This process starts with the explosive exocytosis of the cortical granules, causing the characteristic elevation of the vitelline layer, and is followed by an elaboration of microvilli and subsequent membrane recovery by coated vesicle endocytosis. Chandler and Heuser found that freeze-fracture replicas of eggs fixed with glutaraldehyde and cryoprotected with glycerol before freezing displayed forms of interaction between the granule membrane and the plasma membrane, including particle-free single-bilayer membrane diaphragms formed from apposed fusing membranes, which looked identical to those described by other investigators and proposed as essential intermediates in the process of membrane fusion (see *News and Views* 272, 16; 1978). However such membrane interactions were never found in eggs which had been quick-frozen without glycerination, even after glutaraldehyde fixation. The authors review other recent literature reporting lability of aldehyde-fixed membranes and glycerol-induced membrane changes, and conclude that many of the postulated 'intermediate stages' in fusion are in fact artefacts resulting from the lack of complete stabilisation of lipid bilayers by aldehyde fixation, exacerbated by the dehydrating effects of glycerol. This is further discussed by Chandler in a forthcoming book entitled *Freeze-fracture: Methods, Artifacts and Interpretations* (eds Rash & Hudson, Raven Press, New York). Since glutaraldehyde does not affect most membrane lipids, such lability is not unreasonable, but the question remains as to what chemical changes have already occurred in those membranes to permit such artefacts to develop only in situations where membrane fusion is imminently likely to occur.

The quick-freezing method instantaneously samples one particular moment in a biological process, and avoids the accumulation of intermediate stages



**Fig. 2a**, Characteristic view of the pattern of grouping of surface projections seen on deep-etched postsynaptic membrane fragments. The most obvious pattern is rows four abreast, with many breaks and curves (arrow).  $\times 270,000$ ; so 3 mm  $\approx$  10 nm. **b**, Unidirectional shadow-casting of an unetched E face of the postsynaptic membrane, exposed by fracturing intact, quick-frozen tissue, which illustrates that the shallow bumps seen on this surface also group into curvilinear rows, most often two or four abreast (arrows), like the surface projections in Fig. 2a  $\times 270,000$ ; so 3 mm  $\approx$  10 nm.

**Fig. 3** Diagram illustrating, first, how cleavage of intramembrane receptor molecules could generate the match between the E face lattice and the surface lattice of projections that is observed in etched *Torpedo* post-synaptic membrane; and second, how extraction of whole receptors from one leaflet or the other could generate the large intramembrane particles which also characterise this membrane.



which sometimes happens during slow death aldehyde fixation. As Chandler and Heuser were not able to detect preliminary fusion stages in their quick-frozen fertilised eggs, before seeing fused granules

open to the extracellular space, they concluded that such stages could last no longer than 5 ms. An understanding of the molecular events which initiate membrane fusion thus seems as elusive as ever! □



## 100 years ago

The *Hannoversche Courier* announces that Leibnitz's long-lost calculating machine has been recovered. Leibnitz invented and constructed this machine in 1672, during his stay in Paris. It can add, subtract, divide and multiply and was the wonder of the time. This machine became the property of the Hanover public library, but long ago disappeared from among its treasures. All that was known about its disappearance was that it had once been sent to an instrument maker at Göttingen to be repaired. It has now turned up again in the Göttingen library.

It is only about a year since we gave some account (*Nature*, vol. xviii, p. 361) of the railway bridge which spans the Firth of Tay at Dundee, and on Sunday it was the scene of one of the most terrible railway accidents on record. With the details of this sad occurrence our readers are no doubt familiar; for accurate information as to the prime cause we must await the searching inquiry which will no doubt be instituted. The structure appears to have been subjected to the most rigid tests before being opened to traffic, but we fear there must have been more than one screw loose somewhere. Upwards of 3,000 feet of the high girders are reported to have been swept away. One conjecture is that the train had got well upon the girders when a gust of greater strength had caught the structure. There would thus be, in addition to the ordinary vibration of the train, an enormous lateral pressure from the wind.

From *Nature* 21, Jan. 1, 214; 1880.



# The great mouse festival

from R.J. Berry

To most people the house mouse is a commensal nuisance; to many biologists it is a necessary research tool, bought off the shelf (as it were) in the same way as a chemical. Yet the mouse is a real animal, approved because "its fertility, prolificity, convenient size, short gestation period, its manifold variations, inexpensive maintenance, resistance to open infections, susceptibility to certain diseases, and ease of production conspire to make it the laboratory animal par excellence". So wrote Clyde Keeler in 1931. Ten years later, L.C. Strong was advocating that "... within the near future all research on mice should be carried out on inbred animals or on hybrid mice of known (genetically controlled) origin, where the degree of biological variability has been carefully controlled". As immunological studies developed, this became essential; but as a consequence laboratory mice became more and more removed from their origins.

There are now about 200 highly inbred strains of mice. However the great bulk of these arose from a very narrow base — notably the mouse colony established in Granby, Massachusetts during 1903-15 by Miss Abbey Lathrop, who supplied, among others, W.E. Castle and C.C. Little. The origins of her mice are not known in detail but they included 'fancy' mice imported from various European countries and wild animals from Vermont and Michigan, which were crossed to them. Such widely used strains as the C57 group are descended from the Lathrop colony.

The genetical poverty of established laboratory strains as a result of their small number of ancestors has led in recent years to an interest in wild mice as a source of new inherited variants: the first mouse inborn error of metabolism analogous to a human anomaly (histidinaemia) was discovered in a mouse from Peru; several mice caught five years ago in Taunton were found to have a similar defect in pyruvate kinase to one which produces a clinical anaemia in man. More recently mice from south east Asia (notably *M.m. castaneus* and *M.m. molossinus*) have proved particularly rich in previously unknown variants.

An older line of research in wild mice stems from the widespread occurrence of *t*-alleles in natural populations. Work on the *t*-complex has been stimulated in the past few years because of its linkage to the major histocompatibility complex (*H-2*), with suppression of crossing-over between the two chromosomal segments if a *t*-allele is present. It has been suggested that the *t* and *H-2* segments may represent a duplication, *t* acting as a tissue recogniser before birth and *H-2* taking over post-natally.

Finally, mice have been important to evolutionists because of their apparently very tight social structure, from which it has been argued that the 'effective population size' of mouse populations is less than four individuals, thus permitting considerable genetic drift between generations and providing a paradigm for testing ideas of neutrality and selection acting on particular inherited variants.

All these interests found a focus in a symposium on the house mouse held in London recently\*. It reviewed developments over a wide range of fields from archaeology and taxonomy to gerontology and zoonoses. As such it established hybrid vigour between different disciplines rather than transitory euphoria from exciting experiments. The recurring theme was the problem of identifying the stresses and stratagems of animals exposed in different parts of their range to widely differing environmental conditions. R.J. Berry (Royal Free Hospital Medical School, London) described gene frequency changes of over 15% between cohorts of mice in the same populations living on sub-Antarctic islands, with the two sexes showing changes in opposite directions on South Georgia. No changes occurred in populations living on tropical Pacific islands. But what do these changes mean? J. Klein (University of Tübingen) suggested that the levels of heterozygosity (over 95%) found at the major histocompatibility locus *H-2* (Duncan *et al.* *Nature* 281, 603; 1979) are the result of selection by pathogens acting on varying gene pools established by small numbers of founders. This interpretation depends to some extent on a deme structure of mouse populations allowing local differentiation, and the hitherto generally accepted tightness of this structure was challenged from the point of view of ethological and chemical interactions between individuals by J.H. Mackintosh (Birmingham) and Jane Smith (Pest Infestation Control Laboratory, MAFF).

Implicit in all these approaches was the recognition that both an individual mouse and a mouse population changes in time. This was spelt out by D. Bellamy (Cardiff) who emphasised that many influences are involved in ageing, and it is dangerous to assume that a wild mouse which reaches its physical peak at around 100 days and dies at 400-500 days (even in the laboratory) is responding to the same processes as an inbred animal which may live up to 800 days. A similar point was made by M.E. Jakobsen (North East London Polytechnic) in criticising the naivety inherent in many laboratory studies of physiological responses (particularly brown fat amount and turnover) when compared with the complex and variable

interactions of behaviour and time in mice living in nature.

Notwithstanding, one of the values of the house mouse is the possibility of feedback between knowledge gained of animals living under wild conditions and those kept under controlled conditions in the laboratory. Mary Lyon (MRC Radiobiology Unit, Harwell) reviewed the complexity of gene action in the supergenes on chromosome 17, where embryological analysis of the effects of different loci is a necessary complement to Klein's immunological work. The potential of laboratory animals for interpreting the wild situation (and *vice versa*) was also apparent in contributions from R.C. Roberts (ARC Unit of Animal Genetics, Edinburgh) on the determinants of growth (strong selection for small size in laboratory mice failed to reduce them to the mean size — around 9 gram — of animals from the tropical Pacific) and Jo Peters (MRC Harwell) on protein variation, while J.G.M. Shire (Glasgow) showed how laboratory studies on wild caught mice had influenced — and could enrich — our understanding of endocrine action by providing new structural variants of hormones and by affecting target organ sensitivity.

Surprisingly, the most radical papers came from two taxonomists, J. Marshall (US Natural History Museum) and L. Thaler (Montpellier) who broke the news that the unitary widespread Linnaean species *Mus musculus* with four recognised and 164 invalid subspecies is no more. Thaler spoke merely of species nos 1, 2, 3 and 4 in Europe, but Marshall went the whole taxonomic hog and gave us *Mus musculus, domesticus, spretus* and *abbotti* (= *spicilegus*) in western Europe, *hortulanus* in eastern Europe, *molossinus* in Japan, and *castaneus* as an Asian commensal.

But house mice are even more complicated: traditionally they have had 20 pairs of very uniform acrocentric chromosomes, but local races with 11, 13 and 14 pairs of chromosomes due to centric fusion are now known, and these naturally occurring chromosomal anomalies can be used to produce aneuploid mice, and hence study the pathological effects of aneuploidy (A. Gropp, Lubeck). A new Robertsonian fusion (with 38 chromosomes) from an Orkney island was reported by Klein. No-one knows why these chromosomal races have arisen, and why they persist.

The implications of our knowledge of house mice for ecologists are great: we know more about the behaviour and

\*A symposium on the 'Biology of the House Mouse' was held at the London Zoo on November 22-23, 1979.

genetics of this species(s) than any other. But the real significance of house mouse biology is for biomedical workers: their inbred strains ought no longer to be regarded merely as biochemical artefacts, but as interacting feedback systems dependent on an evolutionary history of adaptation and opportunism. It is pertinent that 13 of the 22 speakers at the Zoo Symposium were employed in Medical Schools or by the MRC.

As medical resources become increasingly stretched, they will have to be applied more consciously at points of need rather than in blunderbuss endeavours to improve the 'environment' (such as water quality, toxicological and dietary hazards, or prenatal care). Wild house mice provide a logical step in interpreting results obtained in the artificiality of the laboratory environment to the confusing heterogeneity of human life. It is to be hoped that the symposium will stimulate both research and funding for work on naturally-living mice. □

## Why ppGpp?

from Andrew Travers

THE ubiquity of purine nucleotides as regulators of macromolecular synthesis was highlighted last summer at a workshop most appropriately dedicated to Fritz Lipmann\*. Historically the identification of regulators such as cyclic AMP, ppGpp, pppAppp, A<sup>5'</sup> pppp<sup>5'</sup> A and 2'5' oligo-A has depended on their unusual and often bizarre structures. Yet in evolution such control systems are unlikely to have arisen *de novo*. Rather it seems more probable that they initially evolved as more effective analogues of pre-existing systems. Such analogues should necessarily be chemically similar to the primordial regulators yet should possess some distinguishing structural features which would allow greater precision in the control of their intracellular concentration.

ppGpp may be considered as a paradigm of such an effector. Its accumulation in bacteria is elicited both by energy starvation and amino acid starvation and, in general, is accompanied by the reduction of protein and RNA synthesis to basal levels. Both genetic (A. Atherley, Iowa State University, Ames; M. Cashel, National Institutes of Health, Bethesda) and biochemical analysis (D. Richter, Universität Hamburg; J. Sy, Rockefeller University) indicate that there are two pathways for its synthesis, one dependent on ribosomes and activated by uncharged tRNA and a second whose activity is apparently independent of protein synthesis. Similarly the degradation of ppGpp to either ppGp or ppG may be equally complex (Richter).

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What is the role of ppGpp? The isolation of a viable mutant which lacks detectable ppGpp clearly demonstrates that the nucleotide is dispensable during normal growth (Cashel). However the ability of the mutant to undergo growth transitions is somewhat impaired. One correlation with the accumulation of ppGpp consequent on environmental stress is a change in transcriptional selectivity manifested as a preferential shut-off of stable RNA production. *In vitro* physiological concentrations of the nucleotide inhibit the initiation of rRNA synthesis with either promoter bound or free RNA polymerase as its target (M. Gruber, Rijksuniversiteit, Groningen; A. Travers, MRC Laboratory of Molecular Biology Cambridge). Yet the extent of inhibition of rRNA synthesis *in vitro* is insufficient to account for the *in vivo* effect. Either the *in vitro* system lacks an activator normally present *in vivo* or ppGpp is not the immediate effector of transcriptional changes following amino acid starvation. Indeed a minor guanine nucleotide, ppGp, was proposed as a candidate true regulator (J. Gallant, University of Washington, Seattle).

Although most evidence favours the hypothesis that ppGpp is a major regulator of macromolecular synthesis during nitrogen starvation analogous changes in gene expression can occur without the concomitant accumulation of this nucleotide. This phenomenon is seen in strains containing mutations in the gene encoding fructose 1:6 diphosphate aldolase (Atherley; Bock & Neidhardt *J. Bact.*, **92**, 470; 1966). This observation again points to the possibility that the response of transcription to environmental stress depends on the balance between the levels of a number of effectors. Such a mechanism would allow diverse means of eliciting the same response. One example of this phenomenon is the process of sporulation in *Bacillus subtilis*. This can be induced by either nitrogen or energy starvation. The latter results in a substantial increase in the intracellular concentration of an adenine nucleotide tentatively identified as pppAppp (H. Rhaese, Universität Frankfurt). By contrast, on nitrogen starvation the GTP pool falls without a concomitant increase in pppAppp levels (E. Freese, National Institutes of Health, Bethesda). In both cases the correlation between the observed phenomena and the physiological response is strong and suggests a causal effect. To reconcile such apparently disparate observations it has to be argued that a fall in the GTP pool is equivalent to a rise in the pppAppp pool. This would be compatible with the opposing functional effects of adenine and guanine nucleotides on RNA polymerase.

Although the appearance and immediate effects of regulatory nucleotides such as ppGpp and pppAppp may be transient there is much evidence to suggest that their long term effects may also be important.

The accumulation of ppGpp is accompanied by the preferential synthesis of two proteins, B56.5 and stringent starvation protein (Reeh *et al. Molec. gen. Genet.* **149**, 279; 1976). At least the latter binds to RNA polymerase and seems to alter its properties in a similar manner to ppGpp itself (Ishihama & Saitoh *J. molec. Biol.* **129**, 517; 1979). Thus ppGpp may induce the synthesis of proteins which act to stabilise the effect which the nucleotide itself elicits. Such a process would constitute a mechanism for changing the state of the cell. In a like manner the 37,000 molecular weight polymerase binding protein which appears in the initial stage of sporulation in *B. subtilis* (Haldewang & Losick *Nature*, **282**, 256; 1979) could stabilise the effects of the transient perturbation of the adenine and guanine nucleotide pools.

In eukaryotes, with the exception of cyclic AMP, there is little evidence for selective regulators of gene expression analogous to ppGpp. In yeast changes in nucleotide pools similar to those observed in *B. subtilis* are correlated with sporulation (Freese; Rhaese). Yet in higher eukaryotes ppGpp and similar compounds are apparently absent (R. Silverman, Iowa State University). Nevertheless highly phosphorylated compounds were reported to accumulate after heat shock in *Drosophila* (Travers) and immediately before sporulation in the water mould *Achyla* (H. Lejohn, University of Manitoba, Winnipeg). Although the appearance of these compounds correlates with changes in macromolecular synthesis there is as yet no conclusive evidence as to whether they have a regulatory role. However adenine nucleotides have been implicated as signals for the initiation of DNA replication. One proposed universal regulator is A<sup>5'</sup> pppp<sup>5'</sup> (P. Plesner, Odense University), which binds tightly to a single subunit of DNA polymerase  $\alpha$  (F. Grummt, Max-Planck Institut für Biochemie, München). Alternatively, the effective signal could be the balance between ATP and ADP in the cell nucleus (E. Rapaport, Harvard University). Such a mechanism would utilise purine nucleotides required for essential intracellular processes, a characteristic which might also be expected to typify early stages in the evolution of regulatory processes.

If adenine nucleotides act as indicators of the energy balance within the cell what is the evolutionary origin of the guanine nucleotide regulators? In bacteria the major physiological role of guanine nucleotides is their involvement in protein synthesis which generates GDP from GTP. ppGpp and related nucleotides could thus be derived from a primitive regulatory system utilising the simple 5' di and triphosphates for coupling transcription and translation. □

\*Workshop on Low Molecular Weight Mediators of Macromolecular Metabolism was held on 24-27 July, 1979 in Hamburg and organised by G. Koch and D. Richter.



## ARTICLES

# Scenario for a warm, high-CO<sub>2</sub> world

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*Plausible patterns for temperature and precipitation changes accompanying a general global warming, such as might occur due to a large increase in atmospheric carbon dioxide levels, are presented. The patterns are determined by comparing the five warmest years in the period 1925–74 with the five coldest in this period. Temperature increases are indicated for most regions, with maximum warming over northern Asia. A few isolated regions show cooling. Precipitation changes are fairly evenly distributed between increases and decreases; the most important features being an increase over India, and decreases in central and south-central USA and over much of Europe and Russia. The latter decreases, should they occur, could have considerable agricultural impact.*

MAN has upset the global carbon cycle by burning fossil fuels and, probably, by deforestation and changing land use. The net result of these activities is to increase the CO<sub>2</sub> content of both the atmosphere and the oceans. Although some details of the carbon cycle remain to be determined<sup>1</sup>, it is generally believed that if fossil fuel use continues to increase at about the rate of increase experienced over the past century then atmospheric CO<sub>2</sub> levels will reach roughly twice today's level by the middle of the next century<sup>2,3</sup>. Furthermore, the enhanced greenhouse effect, could well cause a significant increase in global mean annual surface temperature. Numerical modelling experiments suggest that this increase would be 2° to 3 °C (refs 4–6), a change as great as or greater than any which has occurred in the past 10,000 years. The magnitude of the increase is, however, uncertain and a CO<sub>2</sub>-induced warming could possibly be offset by a natural cooling trend and/or by the effects of various feedback mechanisms (such as that due to possible changes in cloud cover).

Although there is some reason to doubt that a significant warming will occur, its consequences are so great that the possibility must be considered seriously. We will, for the purposes of this article, assume that a global warming will occur. If it does then relatively large shifts in the main atmospheric circulation systems would accompany such a change. These circulation changes will determine the geographical patterns of the changes in temperature, precipitation and winds. Although most regions are expected to warm, the amount of warming must vary considerably from place to place<sup>7</sup>, and it is possible that some regions will become cooler; and although global average precipitation is expected to increase<sup>8–10</sup> some parts of the world will most probably become drier<sup>8,9</sup>. To assess the impact of these changes on society and decide on possible ameliorating courses of action, one must evaluate impacts on a regional scale. It is therefore essential to have some idea of the spatial distribution of future changes in climate. We present here a meteorologically and climatologically self-consistent (that is 'realistic') scenario for the pattern of changes based on recent instrumental records of the natural year-to-year fluctuations in climate.

## Insights into a warm world

Two approaches may be used to derive a scenario for the pattern of climatic changes which might result from a large increase in atmospheric CO<sub>2</sub>. These are: numerical modelling using general circulation models (GCMs)<sup>10–12</sup>; and the use of past warm periods as analogues of the future<sup>8,9</sup>. The latter includes the possibility of using recent instrumental data to determine the characteristics of individual warm years which may then be used as analogues of the future.

Both methods have their limitations. GCMs are restricted by their present state of development; current computer power dictates that these models simulate in detail only one part of the atmosphere–hydrosphere–cryosphere system. Most models only consider the atmosphere and use the hydrosphere and cryosphere as externally specified boundary conditions<sup>11</sup>. GCMs do, however, simulate present-day climate reasonably well; and, provided sea-surface temperatures and ice margins are prescribed, they also appear to simulate ice-age climate in a realistic way<sup>11,13</sup>. For a high-CO<sub>2</sub> world we cannot accurately prescribe sea-surface temperatures and should ideally use a coupled ocean–atmosphere model. Although such models do exist, their simulation of the thermodynamics and dynamics of the oceans is still at a rudimentary level.

Although there is agreement on many of the general results for the high-CO<sub>2</sub> world which have been estimated using GCMs (and other, simpler models), the only spatial detail which has been estimated is the latitudinal variation of changes in temperature<sup>4,6,10</sup>. These results indicate that the greatest temperature changes will occur at high northern latitudes (particularly above 60°N) and in winter. In these respects, expected CO<sub>2</sub>-induced temperature changes show a similar dependence on latitude and season as do the 'natural' fluctuations which have occurred this century: maximum natural variability occurs in winter and in the latitude band 50 to 70°N<sup>14,15</sup>.

The second method is to use the past as an analogue for the future. Four possible candidates have been described by Flohn<sup>16–18</sup>: the mediaeval warm period (about AD 800–1200), the Holocene warm period known as the Hypsithermal or Altithermal (around 4,000 to 8,000 yr BP), the last (Eemian) interglacial (isotope stage 5e, about 120,000 yr BP), and the last period when the Arctic Ocean was believed to be ice free (before 2.5 × 10<sup>6</sup> yr BP). These times are possible analogues for progressively warmer future climates. As Flohn points out, the extent to which these times may be useful as analogues depends in part on how well the ocean and cryosphere boundary conditions which occurred in the past compare to those which might occur in the future. In all four cases, boundary conditions were increasingly different from today. A further difficulty in using any of these periods as analogues is that the available data are quite sparse and are generally indirect and qualitative.

The most detailed analysis of a past epoch as an analogue for a future warmer world is Kellogg's<sup>8,9</sup> analysis of the Hypsithermal. (Sarnthein<sup>19</sup> has also made a detailed study of this period, although not as a warm world analogue.) Kellogg has used published interpretations of pollen data to compile a picture of summer precipitation patterns relative to the present.

Unfortunately, these data are often poorly dated, and what appears to be a synchronous spatial pattern may be the superposition of a number of episodes which all occurred at slightly different times. Nevertheless, Kellogg's picture of global precipitation during the Hypsithermal is widely quoted as an analogue for a high- $\text{CO}_2$  world.

The recent past, for which instrumental records are available, may also be used as a guide to establishing possible future patterns of climatic change. The mean annual surface temperature of the Northern Hemisphere (and quite probably of the whole globe) varies considerably from year to year. Because of this variability one can select individual warm years as analogues for a future warmer world (resulting from increased  $\text{CO}_2$ , or other causes); or, alternatively, use a composite of a number of warm years as a warm world analogue. We have used this method in the present study. A similar approach has been used by Williams<sup>20</sup>, Namias (unpublished) and Drozdov<sup>21</sup> (although this latter work was not directed towards the  $\text{CO}_2$  issue.)

The use of any past records as analogues for a future high- $\text{CO}_2$  world can be criticised on the grounds that past climatic fluctuations were not caused by changes in atmospheric  $\text{CO}_2$  content. Although the causes of year-to-year fluctuations in this century are not clearly understood,  $\text{CO}_2$  is not a possibility. In defence against this criticism we note that numerical model results indicate that, whatever the cause of the warming, there are broad similarities in the patterns of climatic change. The computed effects of, for example, an increase in the solar constant<sup>12</sup> are similar to those due to increased atmospheric  $\text{CO}_2$ <sup>4</sup>. Furthermore, as noted above, what little is known about the latitudinal and seasonal variations in temperature changes which might result from increased  $\text{CO}_2$  indicates that these variations are similar to those for natural year-to-year temperature changes. Essentially, in making use of natural fluctuations

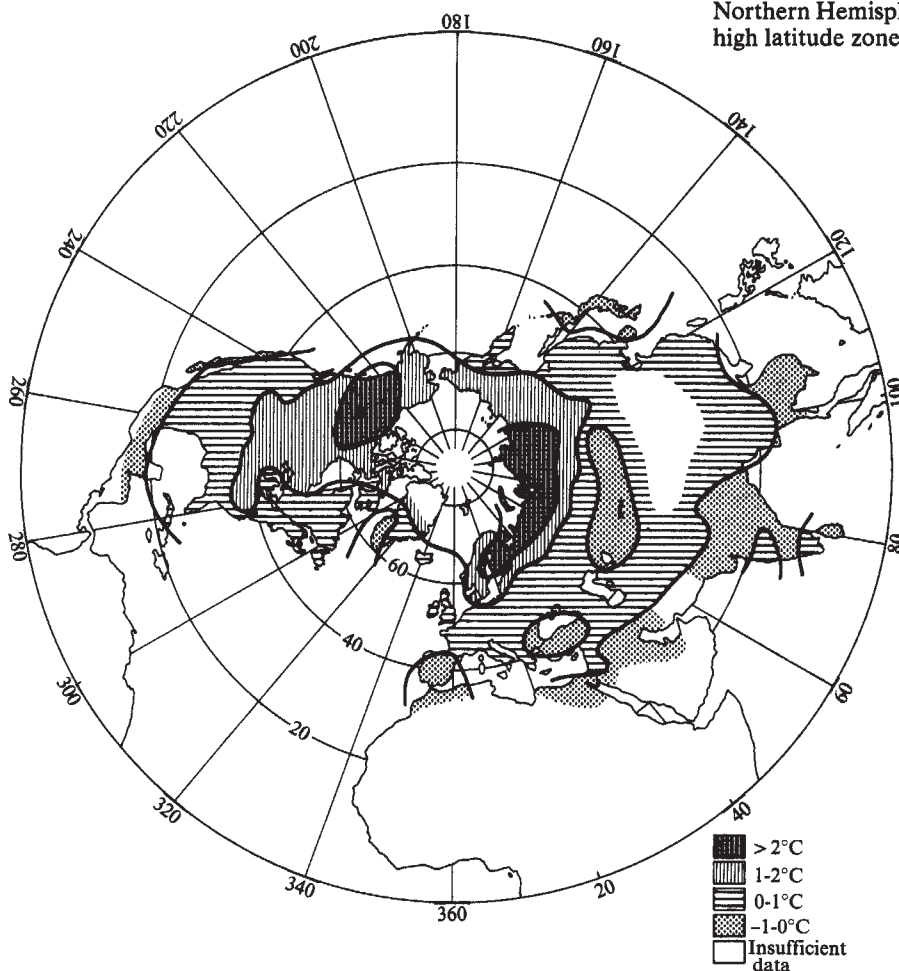
we are assuming that, given similar boundary conditions, there are broad similarities in the way the atmosphere responds to different types of forcing.

### Scenario for a high- $\text{CO}_2$ world

Instead of comparing a group of recent warm years with the long-term mean in order to derive a warm-world scenario, we have used the variations in mean annual surface temperature to maximum advantage by comparing a composite of the five warmest years in the period 1925–74 with a composite of the five coldest years in this period. The period 1925–74 has been chosen because, although temperature data exist before 1925, there are substantial gaps in the coverage and problems of comparability of early and more recent records which make it difficult to rely on the values of, and changes in, spatial means and patterns.

We have chosen to define 'warmest' and 'coldest' in terms of the latitudinal zone  $65^\circ\text{N}$  to  $80^\circ\text{N}$ . This choice is based partly on data availability, but also on the generally accepted belief that  $\text{CO}_2$ -induced changes will be greatest in high northern latitudes. The use of high-latitude data eliminates many of the large data-poor ocean areas. To facilitate averaging over arbitrary regions of the globe, and to produce a homogeneous data series over the whole period, we calculated mean temperatures by first calculating grid point temperatures from station data. Our methods differ slightly from other authors, but our results correlate highly with those of Budyko<sup>22</sup>, Reitan<sup>23</sup>, Angell and Korshover<sup>24</sup> and Borzenkova *et al.*<sup>25</sup>.

For the  $65^\circ\text{N}$  to  $80^\circ\text{N}$  zone the five warmest years are 1937, 1938, 1943, 1944 and 1953, and the five coldest years are 1964, 1965, 1966, 1968 and 1972. The average temperature difference between the warm and cold year groups is  $1.6^\circ\text{C}$  for the high latitude zone, and  $0.6^\circ\text{C}$  for the Northern Hemisphere as a whole, and the years themselves reflect the general warmth of the 1930s and 1940s and the subsequent cooling of the Northern Hemisphere. When the winters are compared (for the high latitude zone), the warm-year group is, on average,  $1.8^\circ\text{C}$



**Fig. 1** Mean annual surface temperature changes from cold to warm years. The corresponding change in the hemispheric mean temperature is  $0.6^\circ\text{C}$ . For reference, the expected change in global mean temperature due to a doubling of atmospheric  $\text{CO}_2$  concentration is  $\sim 2^\circ\text{C}$ .



warmer than the cold-year group; for the summers the corresponding difference is  $0.7^{\circ}\text{C}$ . This greater sensitivity in winter is in accord with expectations for the high- $\text{CO}_2$  world based on numerical modelling results<sup>10</sup>. All of these differences are statistically significant at the 5% level; the annual differences being significant at better than the 0.1% level. This is an important point since it justifies the use of the two groups as analogues of separate populations, even though they have been derived from a single population.

We now find the spatial patterns of temperature and precipitation differences between the cold- and warm-year groups. These patterns (Figs 1 and 2) provide a scenario for (but not a prediction of) the changes in climate which might accompany a  $\text{CO}_2$ -induced global warming. Since modelling results suggest that a doubling of  $\text{CO}_2$  will warm the globe by  $2^{\circ}\text{C}$  or more, the magnitude of  $\text{CO}_2$ -induced temperature changes may be somewhat larger than the differences shown in Fig. 1.

Figure 1 shows the spatial pattern of mean annual surface temperature differences between the cold- and warm-year groups. This map has been constructed from station rather than grid-point data, based on data from 219 stations. Maximum warming occurs in high latitudes and in continental interiors; up to more than five times the hemispheric mean increase in a region extending from Finland across the northernmost parts of Russia and Siberia to about  $90^{\circ}\text{E}$ . Not surprisingly, this area coincides with the region of greatest natural temperature variability. A large part of North America has positive temperature changes of two or more times the hemispheric mean, and this region extends westwards from Alaska, across northern Asia and into Scandinavia. In contrast, some regions show negative differences; Japan, much of India, an area including and adjacent to Turkey, the Iberian peninsula and adjacent North Africa, the south-west coast of the US, a region in central Asia and southwestern Greenland.

An examination of the surface pressure pattern differences between cold and warm years shows that the warm years have intensified high-latitude ( $50^{\circ}\text{N}$  to  $70^{\circ}\text{N}$ ) westerlies, greater cyclonic activity in the arctic and subarctic regions of the eastern hemisphere, and a westward displacement of the Siberian High in the winter half of the year. These features are consistent with the observed temperature differences. The shift in the Siberian High helps to explain the cooling zone in central Asia, which is largely an autumn and winter phenomenon.

Figure 1 only shows differences in mean annual temperature. Seasonal temperature changes are equally important and require further analysis. Most of the large differences which occur at high latitudes, although in the same sense for all seasons, are greatest in winter. In North America there is, in general, warming in all seasons: in the central region north of  $40^{\circ}\text{N}$  the warming is greatest in autumn, while for the rest of the continent (including Alaska) it is greatest in winter. The cooling zone around Turkey is due mainly to cooler springs: summers are generally slightly warmer. In India the cooling is greatest in summer and autumn; in winter and spring there are minor differences of both signs and a considerable degree of spatial variability. Finally, the cooling of the south-west coast of the US is a maximum in summer and the winters are actually warmer.

Figure 2 shows the distribution of precipitation changes from cold to warm years, based on data from 215 stations (most, but not all, being those used in Fig. 1). Precipitation patterns generally have greater small-scale spatial variability than temperature patterns, so the picture presented here is only a guide to broad-scale patterns. When averaged over the Northern Hemisphere we found that the warm-year groups showed a slight (statistically insignificant) increase in annual precipitation of between 1 and 2% compared with the cold-year group. Numerical modelling results indicate that an overall increase of around 5% might accompany a doubling of atmospheric  $\text{CO}_2$ <sup>10</sup>.

The most important features of Fig. 2 are decreases in precipitation over much of the US, most of Europe and Russia (especially France, Spain and the Central Russian Plain) and over Japan, and increases in precipitation over India and the

Middle East. For India, the increases vary from a few per cent in Bangladesh and along the eastern coast, to almost 100% in the north-west. Decreases in precipitation occur in southern India and in the north around New Delhi. In spite of these considerable variations, a clear spatial pattern is apparent, indicative of a more intense monsoon circulation in the warm years. Some of the features in Fig. 2 are the same as those which occurred during the Hypsithermal, the period used by Kellogg<sup>8,9</sup> as an analogue for a future high- $\text{CO}_2$  world. However, there are notable differences. Kellogg shows a wetter Europe and Russia and drier conditions over northwestern Canada, in contrast to our results. Some of these differences probably arise because Kellogg's reconstruction reflects summer precipitation, but the different boundary conditions which prevailed during the Hypsithermal must also be a significant factor.

The seasonal distribution of precipitation changes is important in determining impact. In particular, the consequences of a drier (and warmer) Europe and Russia deserve further consideration. The reductions in rainfall shown in Fig. 2 are not evenly distributed over the year. Largest reductions occur mainly in winter and autumn; but most regions (especially eastern Europe) also show reductions in summer precipitation. A few stations (Lyons, Madrid, Lisbon, Vienna) have greater summer rainfall in the warm years. In the UK annual precipitation changes are minor, except for southern England where precipitation is less in the warm years (spread fairly evenly over winter, spring and summer). For other parts of the UK, although annual changes are small, most places show reductions in spring rainfall and increases in summer rainfall. For the central and western parts of the USA, where there is a reduction in annual precipitation, increases in the winter and spring and decreases in the summer and autumn dominate the picture. Further north, in Canada, summer precipitation increases.

## Changes in boundary conditions

The present results are based on climatic fluctuations which occur on the year-to-year time scale. Climate changes on many time scales (in fact, the effects of increased  $\text{CO}_2$  are relatively slow), and there are important changes which have occurred on time scales too long to be noticeable in the record of the past 50 years. In general, these slow changes have been associated with larger changes in ocean and cryosphere boundary conditions than have been observed this century. Similarly, a large warming at high latitudes will probably lead to changes in Arctic sea ice extent, ocean current systems and sea surface temperatures which are outside the range of variations experienced during the past 50 years. These changes in the boundary conditions for the atmosphere require further discussion since they are central to the use of the past as an analogue and to the use of GCMs in modelling conditions in a warmer world.

It has been suggested that  $\text{CO}_2$ -induced warming may cause seasonal melting of the Arctic sea ice<sup>8,16,27</sup>. How much warming is required for this is uncertain. Manabe's simulation with four times the pre-industrial  $\text{CO}_2$  level<sup>10</sup> showed the sea ice disappearing in summer, and this is commensurate with the work of Budyko<sup>26</sup> and Parkinson and Kellogg<sup>27</sup> which suggests that a summer temperature increase of  $4\text{--}5^{\circ}\text{C}$  in the Arctic Basin would be required to melt the sea ice in summer. These results indicate that sea ice boundary conditions will probably not alter significantly beyond the extremes of the past 50 years at least until the early decades of the twenty-first century: although proposed alterations to the flows of major northward-flowing Russian rivers<sup>28,29</sup> might accelerate Arctic sea ice changes.

We have examined sea ice extent in our cold and warm year groups and found there to be notable differences. Two of the warm years (1937 and 1938) were remarkable for a major reduction in the summer ice along the north coast of Asia and increased outflow of ice from the Arctic in the East Greenland current. Further details are given by Koch<sup>30</sup>.

Sea-surface temperature (SST) patterns form the other main atmospheric boundary condition. In GCM simulations, SSTs are

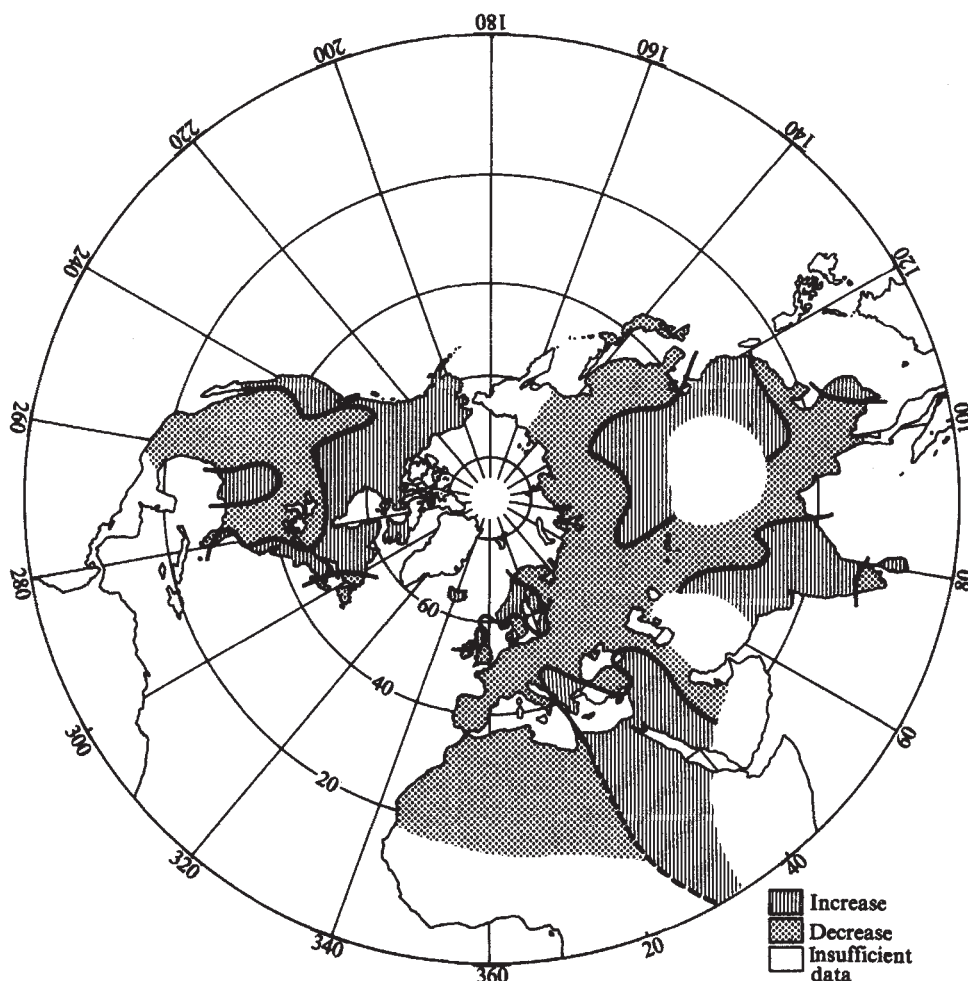


Fig. 2 Mean annual precipitation changes from cold to warm years.

either prescribed (in atmosphere-only models) or determined interactively (in coupled ocean-atmosphere models). Because of data deficiencies we have not been able to examine SST differences between our cold and warm year groups in any detail. We find however, a strong indication that SSTs over most of the North Pacific were cooler in the warm years. Ocean currents are also important, partly because of their relationship with SST patterns, but also because they are responsible for large amounts of latitudinal heat transport. No GCM with realistic ocean currents has been applied to the CO<sub>2</sub> problem. There are insufficient data available to study the details of changes this century, but we note that quite large changes in the Gulf Stream are thought to have occurred in earlier centuries<sup>31</sup>.

Ocean current changes are linked to changes in atmospheric circulation patterns; in particular to changes in surface wind stress. Any significant wind stress reduction would be important because it would result, not only in a change in ocean current dynamics, but also in reduced mixing in the upper layers of the ocean and reduced upwelling, both of which would have profound effects on marine life through changing nutrient supply rates. Budyko and Vinnikov<sup>32</sup> have argued that the mean surface wind stress in a high-CO<sub>2</sub> world might be as little as 50% of today's value. In examining the mean surface wind stress (based on the geostrophic wind) in our warm and cold year groups we found the differences to be small, amounting to only a few per cent and contrasting with the prediction of Budyko and Vinnikov<sup>32</sup>. Although this result is reassuring, further examination of possible regional changes in wind stress is required.

## Conclusions

We have used the word 'scenario' deliberately to distinguish our results from a 'prediction'. A scenario can only provide a guide

to the patterns of climatic change which could occur accompanying a global warming. Our results are limited to relatively small changes in ocean and cryosphere boundary conditions, changes which might well be exceeded in the early decades of next century. Further, as the average hemispheric mean temperature differences between the warm and cold year groups is 0.6 °C, and because changes in regional climate may be non-linear with respect to global or hemispheric temperature change (especially when large changes in boundary conditions are involved), our scenario is most probably only representative until around the turn of the century. It would be dangerous to put any faith in extrapolations which involve hemispheric mean temperature shifts of more than 0.6 °C from the present.

Because the scenario presented here is based on mean conditions for two five-year groups, it is only representative of 5-year to decadal mean conditions. Superimposed on such conditions one must expect a 'normal' amount of year-to-year variability. Whether this variability will be less or more than the present is a controversial issue. Although a link between mean surface temperatures and mean variability may exist, the link is neither simple nor obvious, as van Loon and Williams<sup>33</sup> have shown. We can be sure that the climate will continue to vary considerably from year to year (just as the individual years in our warm and cold groups show noticeable differences), so even in a warm high-CO<sub>2</sub> world there will be regional extremes of warmth and cold, wetness and dryness which will differ markedly from 5 to 10-year mean patterns.

What would be the human (social, economic and political) impact of changes of the type presented here? We can only speculate on some of the possibilities—while noting that impact is at least as much related to the year-to-year variability of climate as it is to the mean or to changes in the mean. First, a substantial increase in the intensity of the Indian monsoon could



have a significant impact. Although the impact of the monsoon in India is generally linked to its failure, increased and more violent precipitation can also have most detrimental effects (as witnessed by the flooding in 1979). Second, in some parts of the world (in particular in Europe and European Russia) temperature increases may occur in association with reductions in precipitation. These two factors will reinforce each other in increasing soil moisture deficits and so tend to have greater agricultural impact than either factor would alone. The climatic differences between warm and cold years which we have shown for Europe and European Russia appear to be of special importance as they indicate that conditions in a high-CO<sub>2</sub> world could be radically different from those given by Kellogg for the Hypsithermal.

We stress that the results presented here are little more than a guide to the type of climatic conditions that are possible in a high-CO<sub>2</sub> world. Should a global warming occur it will, however, certainly affect the weather patterns and climate regimes of the

world and the effects will be different in different regions. These changes will, at the very least, result in shifts of agricultural zones, and some regions will be affected adversely, some favourably. Although some of the effects of a global warming (caused by CO<sub>2</sub> increases, or for any other reason) may well be beneficial<sup>34</sup>, our scenario does show that detrimental effects could occur in some parts of the world, and our work highlights the need for more research into the interdisciplinary problems associated with climatic change which are focused by the current interest in the CO<sub>2</sub> issue.

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# Oxygen and strontium isotope relationships in the British late Caledonian granites

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*Oxygen and strontium isotope data for 25 late Caledonian granite plutons emplaced between ~435 and 390 Myr indicate that the parent magmas were hybrids, derived from partial fusion of mantle-like material and varying proportions of a crustal component.*

COMBINED stable and radiogenic isotopic studies can provide important information concerning both the origin of granite magmas and the post-emplacement history of volcanic and plutonic igneous rocks<sup>1,2</sup>. In this article we present the results of a combined O–Sr reconnaissance isotopic study of twenty-five ~400-Myr-old Caledonian granitic plutons from Scotland and northern England and assess the extent to which crustal anatexis and/or assimilation was important in the generation and subsequent evolution of the parent magmas.

The Caledonian fold belt is thought to have formed as a result of the closure of the 'Iapetus' ocean basin during Lower Palaeozoic times<sup>3</sup>. Granitic magmas were intruded into Pre-

cambrian and Lower Palaeozoic country rocks between approximately 640 and 390 Myr (refs 4, 5). Most important spatially and volumetrically are the late Caledonian granites, the 'Newer' and 'Last' granites of Read<sup>6</sup>. These post-tectonic, largely undeformed granites include those emplaced by active injection into country rock with concordant migmatization and veining (forceful 'Newer' granites) and those emplaced in ring complexes associated with tensional faulting and cauldron subsidence (passive 'Last' granites). Read<sup>6</sup> separates these two classes in time, arguing that deposition of the Lower Old Red Sandstone occurred between emplacement of the forceful 'Newer' and passive 'Last' granites. Apart from those of the north-east Grampian region<sup>4</sup> only a few reliable radiometric ages for the forceful 'Newer Granites' have been published (for example, Strontian, 435 ± 10 Myr)<sup>7</sup> and the oldest reasonably reliable published age for a passive cauldron subsidence granite is that for Lochnagar (415 ± 5 Myr)<sup>5</sup>. However, the temporal classification of Read<sup>6</sup> cannot be applied throughout the British Isles. In Donegal, for example, forceful emplacement (the Main Donegal granite) post-dates passive emplacement (Rosses

**Table 1** Rb-Sr analyses (new) for four Grampian Highland granitoids

| Pluton*         | Rb<br>(p.p.m.) | Sr<br>(p.p.m.) | Rb/Sr<br>(weight) | $^{87}\text{Rb}/^{86}\text{Sr}$<br>(atomic) | $^{87}\text{Sr}/^{86}\text{Sr}$<br>(atomic) | $(^{87}\text{Sr}/^{86}\text{Sr})_i$ † |
|-----------------|----------------|----------------|-------------------|---------------------------------------------|---------------------------------------------|---------------------------------------|
| Ballachulish-I  | 65.72          | 1568           | 0.04193           | 0.1213                                      | 0.70495                                     | 0.70424 ± 4                           |
| Ballachulish-II | 85.88          | 1203           | 0.07141           | 0.2066                                      | 0.70553                                     | 0.70433 ± 6                           |
| Ben Nevis       | 101.1          | 734.2          | 0.1377            | 0.3982                                      | 0.70662                                     | 0.70430 ± 5                           |
| Moor of Rannoch | 62.03          | 1581           | 0.03923           | 0.1135                                      | 0.70548                                     | 0.70482 ± 7                           |
| Garabal Hill    | 94.40          | 638.2          | 0.1479            | 0.4280                                      | 0.70988                                     | 0.70741 ± 7                           |

All analyses utilised isotope dilution and an MM30B mass spectrometer. Uncertainties ( $2\sigma$ ) are  $\pm 0.7\%$  for  $^{87}\text{Rb}/^{86}\text{Sr}$  and  $< \pm 0.01\%$  for  $^{87}\text{Sr}/^{86}\text{Sr}$ . NBS 987 gave a value of  $0.71020 \pm 3$  ( $2\sigma$  for two analyses) at the time of these measurements.

\* Sample locations and ages are given in Table 2.

† Combines estimated uncertainties for  $(^{87}\text{Sr}/^{86}\text{Sr})$  with  $(^{87}\text{Rb}/^{86}\text{Sr})(e^{\lambda t} - 1)$  quadratically; uncertainty in  $t$  (the estimated age) not included;  $\lambda^{87}\text{Rb} = 1.42 \times 10^{-11} \text{ yr}^{-1}$ .

Complex) with all granites emplaced close to 410 Myr (ref. 8 and A. N. H., M. Aftalion and B. E. Leake, unpublished). Similarly, the Fleet pluton of the Southern Uplands in Scotland was emplaced forcefully at  $392 \pm 2$  Myr (ref. 9).

In general, the late Caledonian granites have the calc-alkaline characteristics typical of plutonic rocks emplaced at destructive plate margins<sup>9-11</sup>. The 25 plutons sampled in this study comprise a majority of the late Caledonian granites but do not include the ~460-Myr-old granites studied by Pankhurst<sup>4</sup>. Many of the plutons are composite and zoned. The samples analysed, broadly described as 'granitic', range in composition from diorite and tonalite, through granodiorite, to peraluminous granite with chemical variation continuous among the various rock types. Petrographically and chemically the granitoids span the range from 'sedimentary' to 'igneous' type as defined by Chappell and White<sup>12</sup> with no discernible geographical pattern to their distribution. Recent zircon U-Pb, Rb-Sr, and common Pb reconnaissance studies<sup>5,7,13</sup> have revealed a general pattern of inherited zircon U-Pb memory north of the Highland Boundary Fault, generally low initial Sr isotope ratios ( $< 0.709$ ), and a tendency for Pb to be increasingly radiogenic southwards across the region.

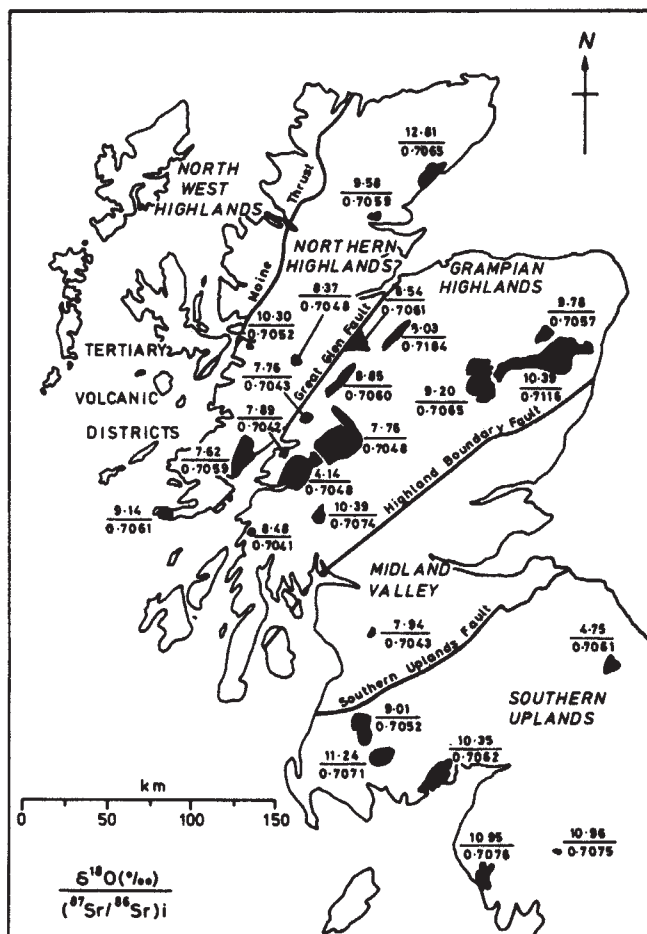
## Isotopic ratio determinations

Oxygen was extracted from whole rock powders by reaction with  $\text{BrF}_3$  and converted to  $\text{CO}_2$  as described by Clayton and Mayeda<sup>14</sup>.  $^{18}\text{O}/^{16}\text{O}$  ratios, determined on the  $\text{CO}_2$  gas using an AWRE Aldermaston isotope ratio mass spectrometer, are reported here in the ' $\delta$ ' notation relative to standard mean ocean water (SMOW) as defined by the Snowbird Quartz (+16.20‰) and African Glass Sand (+9.60‰) standards. Overall precision is 0.1–0.2‰. The Sr isotope ratios presented here are chiefly those reported by Halliday *et al.*<sup>5,9</sup> but include new data (Table 1) for four southwestern Grampian Highlands granites (Ben Nevis, Ballachulish, Moor of Rannoch, Garabal Hill).  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  ratios were calculated from  $(^{87}\text{Sr}/^{86}\text{Sr})$  ratio measurements corrected for  $^{87}\text{Rb}$  decay using independent K-Ar, Rb-Sr and U-Pb ages or from Rb/Sr isochron regression. Samples for which the ages are not accurately known (such as Cluanies) have such low Rb/Sr ratios that the calculated  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  ratios are very close to initial ratios.

The O and Sr isotopic data for this reconnaissance study of the ~400 Myr Caledonian granitoids are presented in Table 2 and, averaged for each pluton, are plotted geographically in Fig. 1. No regional distribution pattern is apparent for either  $\delta^{18}\text{O}$  values or  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  ratios. An area of low  $\delta^{18}\text{O}$  and  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  is present around Glencoe (Ben Nevis–Ballachulish–Moor of Rannoch) and there is a general tendency for the granites of the southwestern Grampian Highlands and Northern Highlands to have slightly lower  $\delta^{18}\text{O}$  values than those of the Southern Uplands and northeastern Grampian and Northern Highlands. Furthermore, no correlation is observed between either  $\delta^{18}\text{O}$  or  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  with distance from the 'Iapetus' suture in the Southern Uplands as proposed by Brown and Hennessy<sup>15</sup>.

Magmas derived from the mantle or a primitive (basal or ultrabasic) mantle-derived source have a characteristic isotopic

composition:  $\delta^{18}\text{O} = 6.0 \pm 0.5\%$  (refs. 16, 17) and  $(^{87}\text{Sr}/^{86}\text{Sr})_i = 0.703 \pm 0.001$  (ref. 18). Fractional crystallisation of a magma may cause slight  $^{18}\text{O}$  enrichment, but should not affect the  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio. However, crustal processes such as weathering, hydrothermal alteration, and metamorphism can produce substantial changes in oxygen isotopic composition. Additionally, the crust has developed a high  $^{87}\text{Sr}/^{86}\text{Sr}$  ratio because of the time-dependent effect of its generally higher Rb/Sr ratios. As a result, the crust is enriched in both  $^{18}\text{O}$  and  $^{87}\text{Sr}$  relative to the mantle and therefore it should be possible to recognise granites produced by crustal melting from those generated in the mantle or lower crust on the basis of their O and Sr isotopic composition. The degree to which a crystallised granite will reflect the



**Fig. 1** Location map showing the physiographical provinces and major tectonic features of northern England and Scotland, the geographical distribution of the 'granites' sampled in this study, and the average  $\delta^{18}\text{O}$  values and initial Sr isotope ratios for each pluton. The proposed 'Iapetus' suture line is considered to trend NE-SW across the Southern Uplands in the vicinity of the Solway Firth.



exact oxygen isotopic composition of the source(s) will depend on several factors, among them the temperature of melting, the degree of partial melting, the degree of volatile loss from the melt, the amount of fractional crystallisation, and the extent of post-emplacement alteration. For example, the extreme  $^{18}\text{O}$  enrichment observed in the Tuscan Igneous Province ( $\delta^{18}\text{O} = 11$  to  $17\%$ )<sup>19</sup> and the high initial  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios characteristic of the ~460-Myr-old Caledonian granites ( $>0.71$ )<sup>4</sup> can only be explained in terms of fusion of large amounts of crustal material. On the other hand,  $^{18}\text{O}$  depletion can result from post-emplacement interaction with meteoric fluids or by partial fusion of materials previously affected by such a process. The  $^{18}\text{O}$  depletion of 5 to 10‰ observed within 2 to 3 stock diameters of most intrusive centres within the Scottish Tertiary Volcanic District<sup>20,21</sup> is typical of such post-emplacement subsolidus exchange.

In Fig. 2 the O and Sr isotopic data for the 25 plutons are shown in histogram form. The samples analysed were, in part, those previously studied by Pidgeon and Aftalion<sup>7</sup> and Halliday *et al.*<sup>5,9</sup> for their U-Pb zircon and Rb-Sr isotopic systematics and thus do not represent a statistically valid sampling of the 'Newer' granites because, in certain instances, multiple samples were taken from individual plutons and, in others, from single outcrops (Table 2). Variations in  $\delta^{18}\text{O}$  and  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  are observed in many of these plutons so that it is difficult to represent the isotopic composition of an intrusion by a single sample<sup>9</sup>. Therefore, in cases for which more than one sample was analysed from a single location (such as Bonar Bridge) or for which the initial Sr isotope ratio was derived from a whole-rock isochron regression on a suite of samples from a particular pluton (such as Lochnagar), only a single, averaged  $\delta^{18}\text{O}$  and  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  value for that pluton has been plotted in Fig. 2. Otherwise, where more than one outcrop of a particular pluton was sampled (as for Doon), each data point is shown.

The large range in both  $\delta^{18}\text{O}$  values (4.1–14.4‰) and  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  ratios (0.7037–0.7184) is apparent. No correlation is observed between either isotopic parameter and emplacement age or intrusion style, except that the two plutons with extremely high  $(^{87}\text{Sr}/^{86}\text{Sr})_{400}$  ratios (Findhorn and Aberdeen) may be significantly older than the other granites sampled. Although the ages for these two plutons are uncertain, their Rb/Sr ratios are sufficiently low that this does not nullify their distinctively high initial Sr isotopic ratios. Substantial within-pluton variation in both  $\delta^{18}\text{O}$  and  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  coincident with compositional trends are observed for those composite plutons studied in detail (Table 1).

No significant differences are observed for either isotopic parameter between the forceful and permitted granites sampled except that the two samples with anomalously low  $\delta^{18}\text{O}$  values Cheviot and Ben Cruachan, are both high-level probably sub volcanic, 'Last' granites. The  $^{87}\text{Sr}/^{86}\text{Sr}$  initial ratios for Moor of Rannoch and Ballachulish (forceful 'Newer' granites) are effectively identical to those of Ben Nevis and Ben Cruachan (passive 'Last' granites). Thus, the recent suggestion by Brown and Locke<sup>22</sup> on the basis of recent trace element and geophysical studies<sup>23–25</sup> that the 'Older' granites, as defined by Read<sup>6</sup>, and the forceful 'Newer' granites are genetically related and largely derived from continental crust whereas the passive 'Last' granites were derived largely from the mantle is incompatible with the isotopic data presented here and the important isotopic work of Pankhurst<sup>26</sup> on the Strontian and Foyers granites.

The generally low  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  ratios and extremely variable  $^{18}\text{O}/^{16}\text{O}$  ratios observed preclude an origin of the forceful 'Newer' granites simply by partial fusion of highly evolved crustal materials as detailed by Pankhurst<sup>4</sup> and Bell<sup>27</sup> for the 'Older' granites and ~460-Myr-old Aberdeenshire granites. Rather, we consider the 'Newer' and 'Last' granites to comprise a distinct magmatic group, as first proposed by Read<sup>6</sup>, which were chiefly formed between ~435 Myr and 390 Myr and that the observed field differences are related to a time-variable local tectonic setting rather than being a genetic characteristic of the granites themselves.

Of particular interest are the two samples which show extremely low  $\delta^{18}\text{O}$  values of  $<5\%$  (Ben Cruachan and Cheviot). These two  $^{18}\text{O}/^{16}\text{O}$  ratios which represent the first recorded occurrence of pre-Tertiary low- $^{18}\text{O}$  granites in the British Isles, are clearly anomalous within the context of the other Late Caledonian granites sampled. Their oxygen isotopic composition can only be explained in terms of either post-emplacement exchange between the granite plutons and  $^{18}\text{O}$ -depleted meteoritic groundwater or a derivation from partial fusion of pre-existing low- $^{18}\text{O}$  crustal rocks.  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  ratios for the two samples show no major enrichment with radiogenic Sr as would be expected if they had originated from a crustal source (Ben Cruachan = 0.7048; Cheviot = 0.7061). Pankhurst *et al.*<sup>28</sup> have shown that Sr isotope ratios are not necessarily affected during hydrothermal interaction which has resulted in substantial  $^{18}\text{O}$  depletion. Although Forester and Taylor<sup>20,21</sup> have documented that the southern porphyritic epigranite of the Tertiary Red Hills complex, Skye was intruded as a low- $^{18}\text{O}$  magma derived from the partial melting of altered metasediments, we favour a post-emplacement cause because both are high-level, subvolcanic centres where the effects of such hydrothermal processes are most likely to be present.

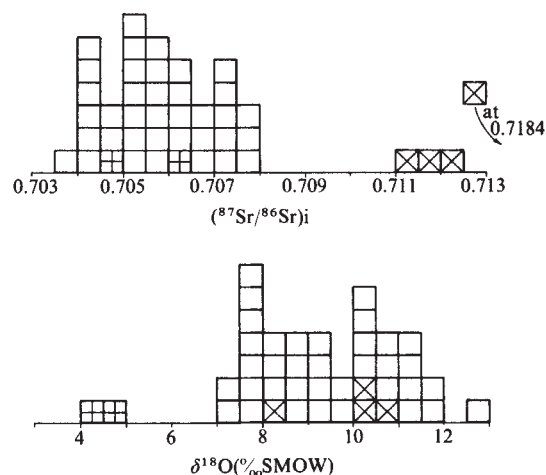


Fig. 2 Histograms of  $\delta^{18}\text{O}$  and  $(^{87}\text{Sr}/^{86}\text{Sr})_i$  for the 'granites' sampled. The two low- $^{18}\text{O}$  samples (Cheviot and Ben Cruachan) are indicated by vertical crosses and the extremely  $^{87}\text{Sr}$ -enriched samples of uncertain age (Findhorn and Aberdeen) by diagonal crosses.

It is also seen from Fig. 2 that approximately one quarter of the granites sampled have  $\delta^{18}\text{O}$  values which lie within Taylor's<sup>16</sup> I and H<sub>1</sub> groups for plutonic igneous rocks. Although the oxygen isotopic composition of these granites is compatible with a mantle or primitive mantle-derived source, allowing for a 1 to 1.5‰ enrichment in  $^{18}\text{O}$  during fractional crystallisation, their initial strontium isotope ratios require that the magmas had to some extent interacted with continental crust during their evolution. Previous O-, Sr-, and Pb-isotopic studies have recognised the involvement of crustal materials in the production of granitic magmas<sup>4,7,16,29–32</sup>. However, the exact nature and degree of this involvement remains unclear. Chappell and White<sup>12</sup> and O'Neil and Chappell<sup>33</sup> have pointed out that it may be possible to recognise granitic rocks derived primarily from metasedimentary crustal sources (S type) and those with primitive igneous origins (I type) on the basis of their petrologic, chemical and isotopic compositions. As regards the Caledonian granites, Pankhurst<sup>26</sup> has pointed out that there is a clear distinction between 'Older' and 'Newer' granites. The 'Older' suite is comprised of small, two-mica granites which have low Ca and Na/K with high  $(^{87}\text{Sr}/^{86}\text{Sr})_i$ , a predominance of biotite over hornblende, and true granitic compositions; all characteristics of an S type affinity. By contrast, the 'Newer' and 'Last' granites are generally large, complex and often composite plutons ranging from diorite to granite in composition, with hornblende, and

high Ca and Na/K with low ( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub>. This would suggest that these late Caledonian granites should be predominantly of I type, but such a categorical classification is not supported by our O and Sr isotopic data.

### Relationship between oxygen and strontium isotope ratios

Figure 3 is a plot of  $\delta^{18}\text{O}$  against ( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub> for the 25 granites analysed. The two low- $^{18}\text{O}$  samples (Ben Cruachan and Cheviot) and the two extremely  $^{87}\text{Sr}$ -enriched samples (Findhorn and Aberdeen) have been omitted. The compositional trends previously described are clearly illustrated. Both  $\delta^{18}\text{O}$  and ( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub> tend to increase sympathetically with the progression from more mafic to more felsic compositions. The  $\delta^{18}\text{O}$  values for this group of late Caledonian granites as a whole appear to form a continuous distribution across the S-I boundary of ~10‰ suggested by O'Neil and Chappell<sup>33</sup> and O'Neil *et al.*<sup>34</sup> for the Australian Palaeozoic granites with no tendency for the forceful 'Newer' granites to be more S type in nature than the passive 'Last' granites.

The most important feature of Fig. 3 is the positive correlation between  $\delta^{18}\text{O}$  and ( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub> which is statistically significant ( $r^2 = 0.75$ ) at the 99% confidence level. That this trend is parallel to that observed by Taylor and Silver<sup>2</sup> for the Peninsular

Ranges batholith of California and Baja would strongly imply that a fundamental petrogenetic process must be responsible for the sympathetic O and Sr isotope variation observed. Differential partial melting of a single source or derivation from a single partial melt at each site are effectively ruled out by the large oxygen isotope variations observed within and between individual plutons. Incidentally, a major difference between the British Caledonides and California/Baja is the striking geographical effects observed in the isotopic systematics of the latter<sup>2</sup>, which is probably due to its simpler geology setting and history.

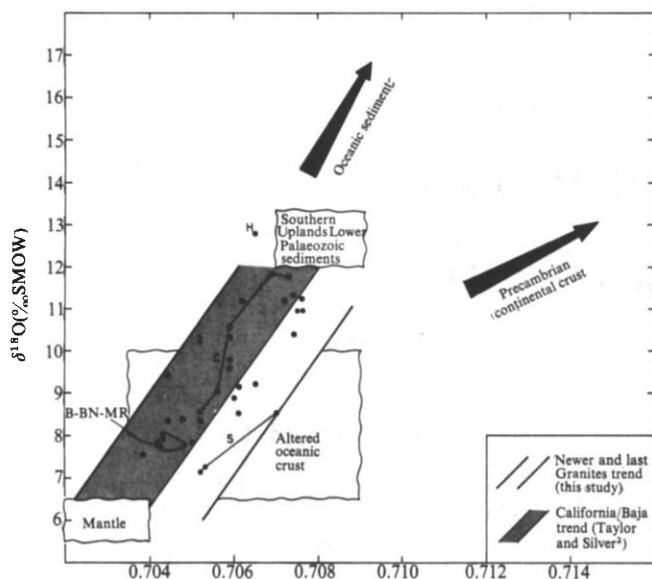
It is also noteworthy that positive correlations of ( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub> with Rb/Sr are observed for the late Caledonian granites which is also suggestive of magma mixing. Halliday *et al.*<sup>5</sup> have considered possible sources for an upper crustal component: Lewisian, Moinian and Dalradian metamorphic rocks and Lower Palaeozoic sediments. Whilst all fulfill the requirements imposed by the granite U-Pb zircon systematics, the Southern Uplands Lower Palaeozoic sediments are the only abundant upper crustal material that could provide a melt derivative of the required Sr isotopic composition. An oxygen isotopic study of these sediments<sup>9</sup>, the result of which is shown in Fig. 3, indicates that such material would be suitable upper crustal end member composition for the magma mixing situation we envisage for the late Caledonian granites. For example, the Shap and Eskdale

Table 2 Oxygen and strontium isotope data for 25 Caledonian granitoids

| Pluton             | Age<br>(10 <sup>6</sup> yr BP) | Locality<br>(Natn. grid. ref.) | Rock<br>type | $\delta^{18}\text{O}$<br>(‰ SMOW) | ( $^{87}\text{Sr}/^{86}\text{Sr}$ ) <sub>i</sub> |
|--------------------|--------------------------------|--------------------------------|--------------|-----------------------------------|--------------------------------------------------|
| Helmsdale          | 420                            | ND 028158                      | lg           | 12.8                              | 0.7065                                           |
| Bonar Bridge       | 400                            | NH 663913                      | lg           | 9.5                               | 0.7059                                           |
| Aberdeen*          | ?                              | NJ 866135                      |              | 10.1                              | 0.7119                                           |
|                    |                                | NJ 906106                      |              | 10.5                              | 0.7120                                           |
|                    |                                | NJ 914077                      |              | 10.5                              | 0.7114                                           |
| Hill of Fare       | 413                            | NJ 711006                      | lg           | 9.2                               | 0.7059                                           |
|                    |                                | NJ 673011                      |              | 9.4                               |                                                  |
|                    |                                | NJ 737033                      | lg           | 11.06/11.08                       |                                                  |
|                    |                                | NJ 728051                      | lg           | 9.4                               |                                                  |
|                    |                                |                                |              | 9.8                               |                                                  |
| Findhorn           | ?                              | NH 726260                      |              | 8.0                               | 0.7184                                           |
| Foyers             | 400                            | NH 548204                      | gd           | 8.5                               | 0.7061                                           |
| Ratagan            | 400                            | NG 901198                      | gd           | 10.3                              | 0.7052                                           |
| Cluanies           | 417                            | NH 175104                      | gd           | 8.4                               | 0.7048                                           |
| Strath Ossian      | 400                            | NM 371809                      | gd           | 8.8                               | 0.7060                                           |
| Lochnagar          | 415                            | NO 340900                      | g            | 9.4                               | 0.7065                                           |
|                    |                                | NO 253863                      | g            | 9.6                               |                                                  |
|                    |                                | NO 260805                      | g            | 8.7                               |                                                  |
|                    |                                | NO 268793                      | g            | 9.1                               |                                                  |
|                    |                                |                                |              | 9.2                               |                                                  |
| Strontian          | 435                            | NM 821608                      | gd           | 7.1                               | 0.7052                                           |
|                    |                                | NM 809533                      | gd           | 7.3                               | 0.7054                                           |
|                    |                                | NM 780650                      | g            | 8.5                               | 0.7070                                           |
| Ben Nevis          | 410                            | NM 115716                      | g            | 7.8                               | 0.7043                                           |
| Ballachulish (I)   | 410                            | NN 025594                      | gd           | 7.8                               | 0.7042                                           |
| (II)               |                                | NN 021589                      | gd           | 8.0                               | 0.7043                                           |
| Moor of Rannoch    | 410                            | NN 465571                      | gd           | 7.8                               | 0.7048                                           |
| Ben Cruachan       | 410                            | NN 039333                      | gd           | 4.1                               | 0.7048                                           |
| Ross of Mull       | 414                            | NM 303230                      | g            | 9.1                               | 0.7061                                           |
| Garabal Hill       | 406                            | NS 306188                      | gd           | 10.4                              | 0.7074                                           |
| Kilmelford         | 427                            |                                | d            | 9.4                               | 0.7044                                           |
|                    |                                |                                | d            | 7.5                               | 0.7038                                           |
| Distinkhorn        | 390                            | NS 599350                      | d            | 7.9                               | 0.7043                                           |
| Cheviot            | 390                            | NT 917164                      | g            | 4.71/4.78                         | 0.7061                                           |
| Doon               | 408                            | NX 467771                      | d            | 8.3                               | 0.7042                                           |
|                    |                                | NX 477787                      | d            | 7.8                               | 0.7050                                           |
|                    |                                | NX 449784                      | gd           | 8.3                               | 0.7052                                           |
|                    |                                | NX 458818                      | g            | 10.2                              | 0.7052                                           |
|                    |                                | NX 455887                      | g            | 10.3                              | 0.7059                                           |
| Criffell           | 397                            | NX 950690                      | gd           | 8.5                               | 0.7052                                           |
|                    |                                | NX 870570                      | gd           | 9.0                               | 0.7056                                           |
|                    |                                | NX 902606                      | g            | 10.6                              | 0.7059                                           |
|                    |                                | NX 900659                      | g            | 11.8                              | 0.7069                                           |
|                    |                                | NX 910660                      | g            | 11.8                              | 0.7073                                           |
| Cairnmore of Fleet | 392                            | NX 548751                      | g            | 11.2                              | 0.7062                                           |
|                    |                                | NX 568727                      | g            | 11.2                              | 0.7072                                           |
|                    |                                | NX 560720                      | g            | 11.3                              | 0.7074                                           |
|                    |                                | NX 570710                      | g            | 11.3                              | 0.7076                                           |
| Shap               | 394                            | NY 555085                      | g            | 11.0                              | 0.7075                                           |
| Eskdale            | 429                            | SD 113944                      | g            | 10.9                              | 0.7076                                           |

\* Strontium isotope initial ratios calculated assuming 400 Myr age, but may be older.





**Fig. 3**  $\delta^{18}\text{O}$ –( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub> relationships for the 'granites' sampled. Not shown are the data for Cheviot, Ben Cruachan, Findhorn and Aberdeen. Isotopic variations corresponding to compositional trends are shown for the 'forceful' granites—Strontian and Criffell. The point indicated by 'H' is the Helmsdale granite and the B–BN–NR field is that for the Ballachulish, Ben Nevis and Moor of Rannoch granites. The shaded trend within the 'Newer Granites' trend is that observed by Taylor and Silver for the Peninsular Ranges batholith in California and Baja. For reference the fields of 'Mantle', 'Altered Oceanic Crust' and 'Southern Uplands Lower Palaeozoic Sediments' are shown together with postulated mixing trends between 'Mantle' and 'Precambrian Continental Crust' and 'Mantle' and 'Oceanic Sediments'.

granites of the Lake District and Fleet of the Southern Uplands plot with high  $\delta^{18}\text{O}$  and ( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub> and lie close to the field of Southern Uplands Lower Palaeozoic sediments shown in Fig. 3. The 'sedimentary' nature of these granites is clear and the conclusion must be drawn that they were largely derived from the geosynclinal metasedimentary wedge in which they occur. However, such sediments do not outcrop north of the Highland Boundary Fault, and even if underthrust during subduction, neither these geosynclinal sediments nor a severely altered oceanic basaltic slab could provide the inherited U–Pb component observed in zircons for granites north of the fault<sup>5,9</sup>. Some clues as to the nature of the crustal component have been provided by recent Pb isotope studies<sup>13</sup> from which it is apparent that a comparatively non-radiogenic Pb component is present in some of the Caledonian granites of the Northern Highlands (for example, Foyers and Ross of Mull). This evidence strongly suggests at least partial derivation of the Northern granites from an old, U-depleted crustal source such as Lewisian granulites. Nevertheless, both the Pb isotope data and the difficulty of extracting large volumes of 'granitic' magmas from granulites further support our contention that mantle or 'new' basic lower

crust was also involved in the production of the late Caledonian granites. The large variations in both  $^{18}\text{O}/^{16}\text{O}$  and ( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub> ratios observed within single plutons (Fig. 3) together with the absence of U–Pb memory in the zircons of the Strontian tonalite contrasted with a marked inherited U–Pb component in the Strontian biotite granite<sup>5,7</sup> endorse this view. Pankhurst<sup>26</sup> has suggested on the basis of trace element and isotopic data that basaltic lower crust is a more likely candidate than mantle peridotite for the low- $^{87}\text{Sr}/^{86}\text{Sr}$  end member.

## Discussion

It thus seems that each of the late Caledonian granites we have studied was derived from a variable mixture of mantle and/or basic lower crust and more isotopically evolved continental material analogous to the local upper crust. Further speculation is difficult because at present there are no oxygen isotope data on Precambrian crustal rocks of Britain other than the unpublished work of Kay<sup>35</sup>. The high  $^{87}\text{Sr}/^{86}\text{Sr}$  ratios of all major Precambrian rock types (Lewisian, Moinean, Dalradian)<sup>5</sup> suggests that such a crustal melt would plot in Fig. 3 with a higher  $^{87}\text{Sr}/^{86}\text{Sr}$  initial ratio for a given  $\delta^{18}\text{O}$  value than the Southern Uplands Lower Palaeozoic sediments and thus not lie on the required mixing line to produce the general  $\delta^{18}\text{O}$ –( $^{87}\text{Sr}/^{86}\text{Sr}$ )<sub>i</sub> trend observed. However, the partial melting of such  $^{87}\text{Sr}$ -enriched Precambrian rocks may be locally important. This would provide an explanation for the tendency of the Strontian data to plot with lower slope than that of Criffell (Fig. 3). It is almost certain that more detailed studies of individual intrusions, such as that recently completed for the three Southern Uplands granites Doon, Criffell and Fleet<sup>9</sup>, will reveal that the overall trend observed in Fig. 3 is comprised of a suite of correlations within the observed limits which relate to the local geological environment.

The model we envisage to explain the isotopic variations observed for the late Caledonian granites involves progressive upward melting from the mantle such that the first magma emplaced is derived from the most primitive source. The rise of granodioritic/tonalitic magma results in the transfer of heat to the surrounding crust sufficient to mobilise acidic melts at high levels in the crust. In some instances successive emplacement of more felsic magmas occurred in discrete pulses after hybridisation, whilst in others the magma pulses became progressively enriched in the crustal component. Simple modelling in a two-component system suggests that between 30 and 80 wt% of crustal contamination is required to account for the observed O and Sr isotopic variations.

This work is a continuation of a large-scale isotopic reconnaissance study of British granites begun by R. T. Pidgeon. We thank Professor H. W. Wilson for his support, J. Borthwick, J. Hutchinson and J. Jocelyn for technical assistance in the laboratory, and M. L. Coleman, S. M. F. Sheppard, W. E. Stephens, L. T. Silver, H. P. Taylor, P. E. Brown, C. Graham, I. Munro and A. Rickard for samples and discussion. The Isotope Geology Unit at SURRC is supported by a grant from NERC. Manuscript typed by Mrs A. McCartney.

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# *E. coli* recA protein-directed cleavage of phage $\lambda$ repressor requires polynucleotide

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*The recA protein mediates both genetic recombination and several cellular responses to DNA damage, including the induction of temperate bacteriophage. Induction of phage  $\lambda$  results from proteolytic cleavage of  $\lambda$  repressor directed by recA protein. We show here that this cleavage reaction requires both polynucleotide and ATP. We suggest that a stoichiometric complex of recA protein and DNA is active both to destroy repressors by proteolytic cleavage and to initiate pairing of this DNA to its homologous sequence in a DNA duplex ('strand invasion').*

THE product of the *recA* gene of *Escherichia coli* appears to have two distinct roles; it is required for all pathways of general genetic recombination<sup>1</sup> and it regulates the expression of several important cellular processes, sometimes called the SOS functions, which are induced when cellular DNA is damaged or its replication is obstructed<sup>2,3</sup>. These processes include the development of temperate bacteriophage, the expression of colicinogenic plasmids<sup>4</sup>, an inhibition of cell division that leads to filamentation, and an error-prone DNA repair capacity that is an important source of radiation and chemical mutagenesis. Furthermore, *recA* protein regulates its own expression: its rate of synthesis is greatly increased by inducing treatments, but this amplification requires *recA* function<sup>5-7</sup>.

How does *recA* protein mediate these different processes? We suggest that it has two separate biochemical activities, which are related by the interaction of *recA* protein with DNA that we describe in this article. Others have shown that purified *recA* protein catalyses the pairing of homologous DNA strands, an activity that accounts for its direct role in genetic recombination<sup>8-11</sup>. We have shown that *recA* protein inactivates the

repressors of phages  $\lambda$  and P22 *in vitro* by proteolytic cleavage, a finding that explains the role of *recA* function in phage induction<sup>12-14</sup>. This activity of *recA* protein to inactivate or modify proteins by proteolytic cleavage may underlie its regulatory function in the expression of other *recA*-dependent processes as well. One specific regulatory role of the proteolytic activity may be to promote the amplification of *recA* protein itself: the *lexA* gene product, thought to be repressor of the *recA* gene<sup>15</sup>, could be inactivated by *recA* protein present at a low basal level before induction, thus causing depression of the *recA* gene<sup>5-7</sup>.

We show here that the activity of purified *recA* protein to direct cleavage of phage  $\lambda$  repressor requires both polynucleotide and ATP. *recA* protein also associates with single-stranded DNA in the set of ATP-dependent reactions that lead to pairing of this DNA to its homologous sequence in a DNA duplex ('strand invasion')<sup>8,10,11</sup>. We suggest that in the presence of ATP, *recA* protein binds in a stoichiometric complex with single-stranded DNA, and is thereby activated both to attack repressors and to engage in DNA strand pairing.

## Repressor cleavage depends on ATP and polynucleotide

We have reported that purified *recA* protein promotes the specific proteolytic cleavage of purified  $\lambda$  repressor into two fragments, in a reaction that requires ATP and small molecules<sup>12,13</sup>. The requirement for polynucleotide originally was obscured by the presence of a contaminating polynucleotide synthetic activity in *recA* protein prepared as we previously described. Because this activity is inhibited by inorganic phosphate and because it sediments at about 9S (data not shown), it is probably polynucleotide phosphorylase, which utilises ADP derived from ATP to synthesise polyribadenylic acid<sup>16</sup>. The experiment of Fig. 1 shows that  $\lambda$  repressor cleavage directed by highly purified *recA* protein is dependent on both ATP and polynucleotide, here denatured  $\lambda$  DNA. The ATP analogue ATP- $\gamma$ S<sup>20</sup> substitutes effectively for ATP and supports a several-fold higher rate of cleavage than does ATP. For this and all experiments reported here we used *recA* protein prepared from cells carrying the *recA* mutation *tif*<sup>-</sup> (ref. 15), although we obtained qualitatively similar results using *recA* protein from *tif*<sup>+</sup> cells.

A variety of single-stranded polynucleotides support the cleavage of repressor by *recA* protein; native DNA is much less effective, particularly in reactions at higher salt concentrations (Table 1). Other polynucleotides that are active include denatured DNA restriction fragments several hundred nucleotides long, single-stranded DNA of phage G4, poly (rC) and poly(U) (data not shown). We also have detected cleavage in the presence of oligonucleotides as short as six nucleotides in the ATP- $\gamma$ S dependent reaction (data not shown). The finding that poly(rA) stimulates cleavage supports our suggestion that less purified *recA* fractions lack the polynucleotide requirement because poly(rA) is synthesised during the cleavage reaction. Because homopolymers are active, the formation of double-stranded polynucleotide<sup>9</sup> is not essential to the reaction.

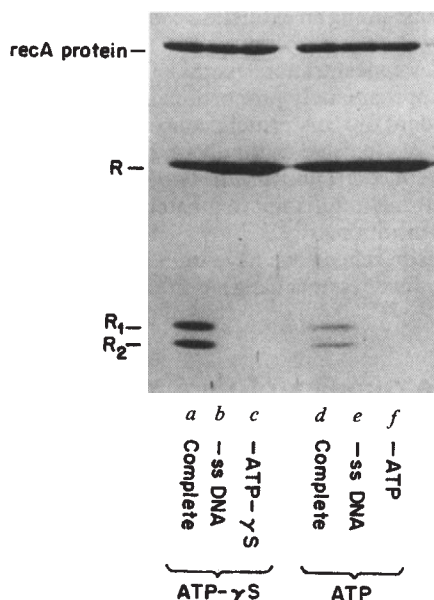
**Table 1** Rate of *recA* protein dependent cleavage of  $\lambda$  repressor in the presence of various polynucleotides

| Polynucleotide             | Polynucleotide concentration<br>( $\mu\text{g ml}^{-1}$ ) | Rate of cleavage<br>(ng per min per $\mu\text{g recA}$ protein) |                       |
|----------------------------|-----------------------------------------------------------|-----------------------------------------------------------------|-----------------------|
|                            |                                                           | Low salt<br>(0.06 M)                                            | High salt<br>(0.18 M) |
| 1. None                    | —                                                         | <0.1                                                            | <0.1                  |
| 2. Denatured $\lambda$ DNA | 1.5                                                       | 38                                                              | 12                    |
| 3. Native plasmid DNA      | 22                                                        | 7.2                                                             | 0.3                   |
| 4. poly(rA)                | 1.0                                                       | 50                                                              | 19                    |
| 5. poly(dT)                | 1.5                                                       | 43                                                              | 10                    |
| 6. (dA) <sub>16</sub>      | 6.0                                                       | 25                                                              | 4.0                   |
| 7. (dT) <sub>9</sub>       | 20                                                        | 25                                                              | 4.9                   |

Reaction mixtures of 20  $\mu\text{l}$  were prepared as described in Fig. 1, using 1 mM ATP- $\gamma$ S, 2 mM  $\text{MgCl}_2$ , 3.4  $\mu\text{g}$  repressor, 0.6  $\mu\text{g}$  *recA* protein, polynucleotide as indicated, and salt as indicated. 'Low salt' was 0.015 M NaCl and 0.045 M KCl; 'high salt' was 0.135 M NaCl and 0.045 M KCl. After incubation for 45 min at 37 °C (low salt reaction) or 2 h at 37 °C (high salt reaction) cleavage was determined as described in Fig. 3. Homopolymers and oligonucleotides were obtained from P-L Biochemicals. Plasmid DNA was a derivative of pMB9, made linear by digestion with *EcoRI* endonuclease followed by phenol extraction.



The rate of repressor cleavage in the ATP- $\gamma$ S stimulated reaction is approximately constant for several hours (Fig. 2). In optimal conditions (Table 1) the rate of repressor cleavage is 40–50 ng repressor per min per  $\mu$ g recA protein; ATP provides about one-fourth this rate. Thus recA protein promotes cleavage of an equimolar amount of repressor in about 15 min in



**Fig. 1** Dependence of the recA protein directed cleavage of  $\lambda$  repressor on polynucleotide and ATP or ATP- $\gamma$ S. Reaction mixtures of 30  $\mu$ l contained 13 mM Tris HCl pH 7.5, 1 mM potassium phosphate, 0.55 mM EDTA, 0.4 mM  $\text{CaCl}_2$ , 2.4 mM dithiothreitol, 0.145 M NaCl, 0.015 M KCl, 5% (w/v) sucrose, 4% (v/v) glycerol, 0.9  $\mu$ g recA protein, 3.2  $\mu$ g  $\lambda$  repressor, and 1.3  $\mu\text{g ml}^{-1}$  heat-denatured  $\lambda$  DNA (unless omitted). Reactions a–c contained in addition 2 mM  $\text{MgCl}_2$  and 1 mM ATP- $\gamma$ S (unless omitted); reactions d–f contained in addition 10 mM  $\text{MgCl}_2$  and 5 mM ATP (unless omitted). Track a, complete reaction (ATP- $\gamma$ S); track b, DNA omitted; track c, ATP- $\gamma$ S omitted; track d, complete reaction (ATP); track e, DNA omitted; track f, ATP omitted. After incubation for 60 min at 37 °C reaction mixtures were analysed by SDS-gel electrophoresis<sup>12</sup> and staining with Coomassie brilliant blue. Bands corresponding to recA protein,  $\lambda$  repressor, and both fragments derived from  $\lambda$  repressor are indicated<sup>12</sup>. *tif*<sup>–</sup>-recA protein was prepared from *E. coli* DM1187 as described in ref. 13 with the following changes. The ammonium sulphate pellet obtained by fractionating the polymin P eluate was extracted with buffer to which 0.20 g  $\text{ml}^{-1}$  ammonium sulphate was added; after centrifugation, protein was precipitated from the supernatant by addition of 0.15 g ammonium sulphate per ml. After dialysis, this protein was chromatographed on DNA-agarose, or on phosphocellulose, or on phosphocellulose in 20 mM potassium phosphate, pH 6.5, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol, eluting with a gradient of KCl to 0.25 M in the same buffer<sup>9</sup>. Usually we used two cycles of phosphocellulose chromatography, or DNA-agarose followed by phosphocellulose. The peak of protein was concentrated with ammonium sulphate and centrifuged on a sucrose gradient as described in ref. 13, except that the gradient was collected from the top to avoid contamination with polynucleotide phosphorylase, which sediments faster than recA protein. The protein appeared homogeneous by gel electrophoresis in SDS. The main criterion for purity was complete dependence on added polynucleotide for repressor cleavage, a condition that correlated with very low or undetectable activity of polynucleotide phosphorylase. Phosphate was added to reaction mixtures to inhibit any residual polynucleotide phosphorylase. There was no detectable endonuclease active on denatured  $\lambda$  DNA in either recA protein or  $\lambda$  repressor.  $\lambda$  repressor was prepared from *E. coli* 294 (pKB277) (ref. 17) by the following modification of the method of Sauer and Andreegg<sup>18</sup>. Cells were grown in medium containing 16 g tryptone, 10 g yeast extract, and 5 g NaCl per litre to an  $A_{550}$  of 1. After fractionation of the crude extract with polymin P and ammonium sulphate, protein was loaded onto a single stranded DNA-agarose column prepared as described by Schaller *et al.*<sup>19</sup>, in a buffer containing 10 mM Tris HCl pH 7.9, 1 mM EDTA, 2 mM  $\text{CaCl}_2$ , 3 mM dithiothreitol, 5% glycerol (v/v), and 0.1M KCl. Protein was eluted with the same buffer containing 0.4 M KCl, chromatographed on hydroxylapatite<sup>13</sup>, concentrated with ammonium sulphate, and centrifuged on a 10–30% (v/v) glycerol gradient in the above buffer containing 10 mM dithiothreitol and 0.2 M KCl for 40 h at 35,000 r.p.m. in the Beckman SW41 rotor. Sometimes the centrifugation was repeated to remove traces of polynucleotide phosphorylase.  $\lambda$  DNA, prepared by phenol extraction, was denatured by heating for 5 min at 100 °C, followed by rapid chilling. ATP- $\gamma$ S was obtained from Boehringer.

these conditions. Both recA protein and repressor are present at concentrations that limit the rate of this reaction<sup>14</sup>. From the experiment of Fig. 3, track a, it can be seen that recA protein functions catalytically: 0.9  $\mu$ g recA protein directed cleavage of 6  $\mu$ g repressor, representing a turnover of 10 repressor monomers (molecular weight (MW) 26,000) per recA monomer (of MW 40,000). Figure 3, track c demonstrates that essentially all of the input repressor is sensitive to recA protein directed cleavage.

A parenthetical, but important point is that the further purification of recA protein does not separate from it any factors required for repressor cleavage. Thus we presume that recA protein is the protease, although we lack a rigorous proof that recA protein does not act in concert with a protease present in trace amounts that we cannot detect separately. We can conclude that recA protein is directly required for the reaction, because variants of recA protein prepared from mutants display different activities for repressor cleavage *in vitro* (refs 12, 13 and unpublished experiments).

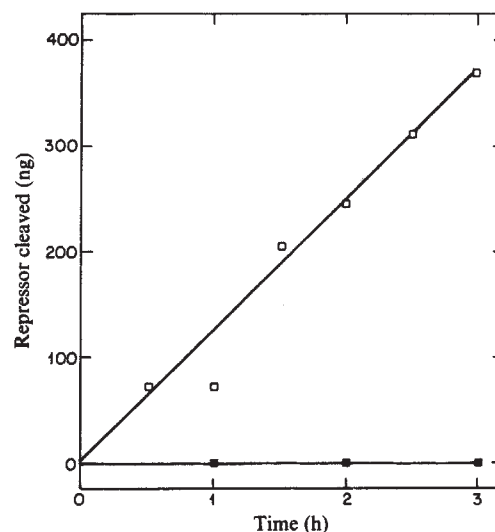
## The polynucleotide requirement for repressor cleavage

Since both recA protein and  $\lambda$  repressor are DNA-binding proteins, either or both might interact with polynucleotide in repressor cleavage. The following results suggest that the primary interaction of polynucleotide is with recA protein.

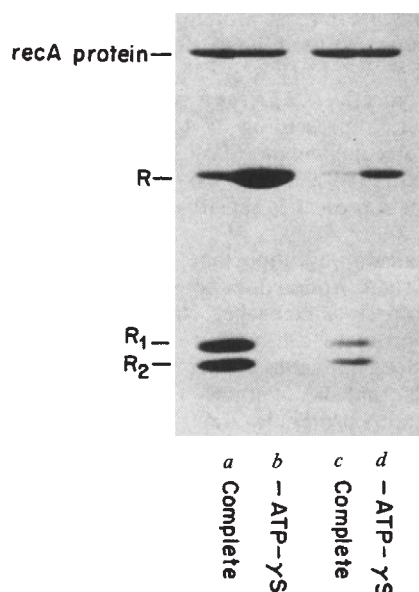
### Polynucleotide-dependent ATPase activity of recA protein:

Purified recA protein has a polynucleotide-dependent ATPase activity; its dependence on single-stranded DNA is shown in Fig. 4. The characteristics of this ATPase suggest that the same interaction of polynucleotide and ATP with recA protein activates both ATPase and the repressor cleavage reaction. All single-stranded polynucleotides that stimulate repressor cleavage (Table 1) also stimulate the ATPase, except the two oligonucleotides. (These oligonucleotides stimulate repressor cleavage with ATP- $\gamma$ S, but not detectably with ATP; data not shown.) Native DNA is severalfold less effective than denatured DNA in stimulating the ATPase in these conditions (data not shown).

The ATP analogue ATP- $\gamma$ S, which substitutes effectively for ATP in repressor cleavage, is not hydrolysed by recA protein; in



**Fig. 2** Time course of  $\lambda$  repressor cleavage. Reaction mixtures of 20  $\mu$ l were prepared as described in Fig. 1, but containing 0.14 M NaCl, 0.045 M KCl, 2 mM  $\text{MgCl}_2$ , 1 mM ATP- $\gamma$ S, 0.5  $\mu$ g recA protein, 4.1  $\mu$ g  $\lambda$  repressor, and 1.4  $\mu\text{g ml}^{-1}$  denatured  $\lambda$  DNA (unless omitted). After incubation reaction mixtures were analysed by SDS gel electrophoresis and staining with Coomassie brilliant blue. The amount of repressor cleaved was measured by densitometric tracing of fragments  $R_1$  and  $R_2$ , using as a standard repressor fragments from a reaction mixture in which a known amount of repressor was completely cleaved (as in Fig. 3c). ■, DNA omitted; □, complete reaction.



**Fig. 3** Efficiency of  $\lambda$  repressor cleavage. Reaction mixtures of 30  $\mu$ l were prepared as described in Fig. 1, except that they contained 2 mM  $\text{MgCl}_2$ , 1 mM ATP- $\gamma$ S (unless omitted), 0.9  $\mu$ g recA protein, 1.3  $\mu$ g  $\text{ml}^{-1}$  denatured  $\lambda$  DNA and  $\lambda$  repressor as given. After incubation for 5 h at 37  $^\circ\text{C}$ , reaction mixtures were analysed as in Fig. 1. Track a, complete reaction with 8.4  $\mu$ g  $\lambda$  repressor; track b, as track (a), but ATP- $\gamma$ S omitted; track c, complete reaction with 1.4  $\mu$ g  $\lambda$  repressor; track d, as track c, but ATP- $\gamma$ S omitted.

fact, it is a potent inhibitor of both the ATPase activity (Fig. 4) and the strand invasion reaction<sup>10,11,13</sup>. A reasonable interpretation of these facts is that binding alone of either nucleotide to a site on recA protein in the presence of polynucleotide activates repressor cleavage, whereas hydrolysis of ATP is required for strand invasion. It is consistent with this proposal that for both repressor cleavage and ATPase, ATP is required at a relatively high concentration and ATP- $\gamma$ S is effective at a relatively low concentration. ATP is required at  $\sim 1$  mM to stimulate repressor cleavage, near its  $K_m$  for the ATPase reaction ( $\sim 0.5$  mM) (data not shown). ATP- $\gamma$ S inhibits the ATPase reaction in the micromolar concentration range (Fig. 4), at which it is also effective in supporting repressor cleavage.

**Polynucleotide concentration dependence of repressor cleavage:** The rate of recA protein-directed cleavage of repressor depends on the concentration of polynucleotide in the reaction mixture. When ATP- $\gamma$ S is used, there is a striking polynucleotide concentration optimum. Figure 5 shows a polyacrylamide gel analysis of reaction mixtures incubated with increasing concentrations of single-stranded DNA. In control experiments, we found the rate of repressor cleavage to be constant during this incubation, so that we can infer the rate of cleavage from the final yield of repressor fragments  $R_1$  and  $R_2$  (see Fig. 2). It is apparent that a maximum rate is reached as the concentration of DNA is increased and that higher concentrations sharply inhibit the reaction. Single-stranded DNA of phage G4, poly(rA), and poly(dT) yield similar concentration optima and maximum cleavage rates. The optimum for duplex DNA is much broader and occurs at a higher DNA concentration. When ATP is used instead of ATP- $\gamma$ S, the concentration optimum for single-stranded polynucleotide is the same, but the inhibition by higher concentrations is less pronounced.

The nature of the DNA concentration dependence of repressor cleavage suggests that the polynucleotide interacts with recA protein: both the maximum rate of cleavage and the DNA concentration at which this maximum occurs vary with the concentration of recA protein. To show this, we have constructed DNA dose curves similar to that in Fig. 5, using three different concentrations of recA protein. From this experiment (Fig. 6) we draw three conclusions:

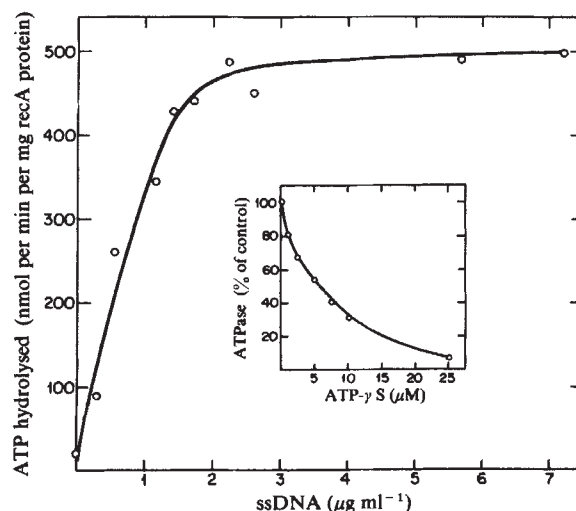
- (1) The DNA concentration optimum is approximately proportional to the recA protein concentration. Thus with 15, 30 and 60  $\mu\text{g ml}^{-1}$  recA protein, the respective DNA concentration optima are about 0.8, 1.5 and 3  $\mu\text{g ml}^{-1}$ . Taking 40,000 and 330 as the molecular weights of a recA monomer<sup>6</sup> and a nucleotide of DNA, we calculate that the maximum rate of cleavage occurs with six nucleotides per recA monomer. (The major uncertainty in this calculation is the concentration of recA protein, which we estimated by assuming an extinction coefficient of 1.0  $\text{mg}^{-1} \text{ml}^{-1}$  at 280 nm.)
- (2) At a DNA concentration less than the optimum, the rate of cleavage is approximately proportional to DNA concentration and independent of recA protein concentration.
- (3) The rate of cleavage at the DNA optimum increases with recA protein concentration; for the two higher concentrations of recA protein, this rate is approximately proportional to recA protein concentration.

In similar experiments we have found that the DNA concentration optimum does not change as the repressor concentration is varied.

## The active complex of recA protein and DNA

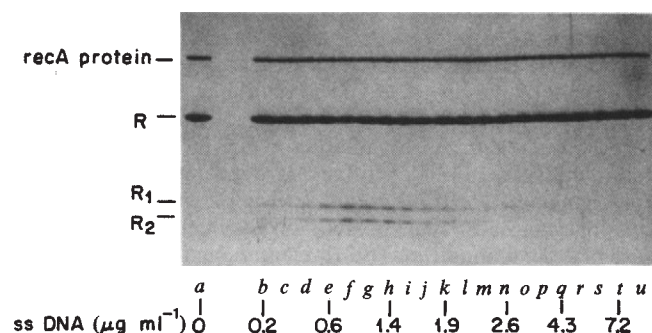
Because the greatest rate of repressor cleavage occurs at a constant ratio of recA protein to DNA for various concentrations of recA protein, we infer that these components form a stoichiometric complex in the presence of ATP or ATP- $\gamma$ S that is active in cleavage. Others have suggested that recA protein binds DNA tightly in the presence of these nucleotides<sup>10,11</sup>. A reasonable presumption is that binding to polynucleotide causes a conformational shift in recA protein to a form that is active to bind and cleave repressors. In this model, the function of ATP or ATP- $\gamma$ S in repressor cleavage might be to promote the formation of the active complex of recA protein and polynucleotide. The repressor also could contact polynucleotide during the cleavage reaction, but we have no information about this possibility.

It seems likely that the recA protein-DNA complex active in repressor cleavage is the same that is active in the strand



**Fig. 4** DNA-dependent ATPase activity of recA protein. (Inset: inhibition of recA ATPase by ATP- $\gamma$ S). Reaction mixtures contained 11 mM Tris-HCl, pH 7.5, 0.18 M NaCl, 10 mM  $\text{MgCl}_2$ , 0.5 mM dithiothreitol, 0.35 mM EDTA, 5 mM [ $\gamma$ - $^{32}\text{P}$ ]ATP, 6.5% (w/v) sucrose, 29  $\mu\text{g ml}^{-1}$  recA protein, and denatured  $\lambda$  DNA as indicated. After an incubation of 15 min at 37  $^\circ\text{C}$ , ATP hydrolysis was determined by the method of Avron<sup>22</sup>. Inhibition by ATP- $\gamma$ S was measured in reaction mixtures as above, but containing 27  $\mu\text{g ml}^{-1}$  recA protein, 68  $\mu\text{g ml}^{-1}$  denatured DNA, 1.5 mM [ $\gamma$ - $^{32}\text{P}$ ]ATP, and ATP- $\gamma$ S as indicated; ATP hydrolysis was determined after an incubation of 30 min at 37  $^\circ\text{C}$ .





**Fig. 5** Effect of single-stranded DNA concentration on repressor cleavage. Reaction mixtures of 20  $\mu$ l were prepared as described in Fig. 1, except that they contained 0.14 M NaCl, 0.04 M KCl, 2 mM KCl (ref. 2), 1 mM ATP- $\gamma$ S, 0.5  $\mu$ g recA protein, 3.7  $\mu$ g  $\lambda$  repressor, and denatured  $\lambda$  DNA as indicated. After incubation for 2 h at 37°C reaction mixtures were analysed by SDS-gel electrophoresis and staining with Coomassie brilliant blue. Track a, no DNA; tracks b–u, 0.2, 0.3, 0.4, 0.6, 0.8, 1.2, 1.4, 1.5, 1.7, 1.9, 2.2, 2.5, 2.6, 3.0, 3.5, 4.3, 5.0, 5.7, 7.2 and 9.5  $\mu$ g ml<sup>-1</sup> DNA.

invasion reaction. Shibata *et al.*<sup>8</sup> have determined an optimum ratio of one recA monomer to five nucleotides of single-stranded DNA to catalyse invasion of this strand into duplex DNA to form a D-loop; this is identical within experimental error to our calculated optimum of six nucleotides per recA monomer for the repressor cleavage reaction. Furthermore, D-loop formation also is inhibited if DNA is present in excess over the apparent stoichiometric value<sup>8,10</sup>. An obvious difference between the reactions is that ATP- $\gamma$ S promotes repressor cleavage but not D-loop formation. However, Cunningham *et al.*<sup>11</sup> have shown that ATP- $\gamma$ S does stimulate recA protein to melt duplex DNA in the presence of single-stranded DNA. Thus recA protein is activated both for repressor cleavage and for this initial step of strand invasion by interaction with single-stranded DNA and ATP- $\gamma$ S. (Presumably ATP hydrolysis is required for a subsequent step of strand invasion.)

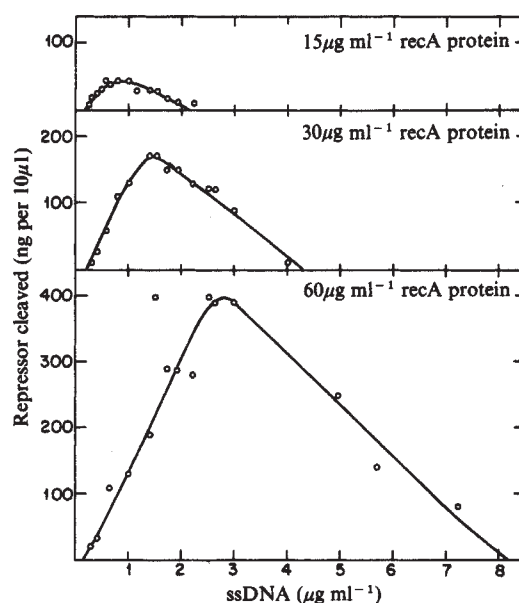
We do not know why excess DNA inhibits the cleavage and strand pairing activities of recA protein. We note that inhibition of repressor cleavage by excess DNA is most striking in the presence of the ATP analogue ATP- $\gamma$ S, which is not hydrolysed, and that the ATPase activity of recA protein is not inhibited by high concentrations on DNA. One possibility is that excess DNA interacts with a second site of recA protein normally utilised in the strand pairing reaction, and that this interaction locks the protein into an inactive form when the non-hydrolysable analogue is present. As excess single-stranded DNA also inhibits strand invasion, it probably affects recA protein rather than repressor.

## Regulation of recA protein activity *in vivo*

A lysogen is induced by treatments that damage cellular DNA, leading to cleavage and inactivation of repressor by recA protein. We have described conditions in which recA protein cleaves  $\lambda$  repressor *in vitro*. How is this activity of recA protein regulated to cause induction? The presence of wildtype recA protein in the cell is not sufficient to cause repressor inactivation *in vivo*. Instead, it is likely that a signal invoked by damage to cellular DNA activates recA protein to cleave repressors<sup>5–7,23</sup>. This activation also is thought to cause amplification of recA protein: by hypothesis, the inducing signal stimulates a small amount of recA protein present before induction to cleave a repressor of the *recA* gene, encoded by the *lexA* gene, leading to amplification of the recA protein itself. Both the activation and the increased amount of recA protein are probably required for full expression of *recA*-dependent processes, including phage repressor inactivation.

How can we relate the requirement for a signal to activate recA protein *in vivo* to the fact that recA protein is active in our conditions *in vitro*? One possibility is that we are providing the signal, or one possible signal, by adding single-stranded DNA. Many inducing treatments (such as UV irradiation) lead to single-stranded regions ('gaps') in chromosomal DNA because replication is interrupted at lesions (such as pyrimidine dimers)<sup>24</sup>; such gaps could bind and activate recA protein and thus be a sufficient signal for induction. It is reasonable that recA protein should be directed to gaps, because its major DNA repair function is probably to remove such discontinuities by provoking recombination of the damaged helix with an intact sister chromatid. Alternatively, oligonucleotides resulting from degradation of damaged DNA could provide a signal to activate recA protein<sup>23</sup>.

We doubt, however, that a polynucleotide derived from damaged DNA is the only possible signal. A mutation in the *recA* gene, named *tif*<sup>25</sup>, alters the protein to make it active without damage to cellular DNA; it is sufficiently active at high temperature (40°C) to allow full induction of *recA*-dependent functions, and it has substantial activity at low temperature (30°C) in the absence of an inducing treatment<sup>15</sup>. Yet purified *tif*-recA protein, which we used in these experiments, is completely dependent on polynucleotide for repressor cleavage activity *in vitro*. What polynucleotide does *tif*-recA protein utilise *in vivo*? One possibility is duplex chromosomal DNA, since native DNA has significant activity to promote repressor cleavage *in vitro* (Table 1). Alternatively *tif*-recA protein could interact with a unique DNA structure at a growing fork or at the origin of replication<sup>26,27</sup>. In either case, the *tif*-recA protein might be able to utilise chromosomal DNA under conditions in which the wild type protein cannot. The fact that *tif*-recA protein appears to utilise some undamaged polynucleotide in the cell suggests a second possible type of inducing signal for recA protein: a regulatory molecule could be elaborated in induced cells that binds recA protein and allows it to interact with chromosomal DNA, so that recA protein is activated both to cleave repressors and to engage in recombination. Further study of the behaviour of recA protein *in vitro* may reveal what signal or signals activate it *in vivo*.



**Fig. 6** DNA dependence of repressor cleavage at various concentrations of recA protein. Reaction mixtures prepared as in Fig. 1 contained 0.14 M NaCl, 0.045 M KCl, 2 mM MgCl<sub>2</sub>, 1 mM ATP- $\gamma$ S, 120  $\mu$ g ml<sup>-1</sup>  $\lambda$  repressor, and recA protein and denatured  $\lambda$  DNA as indicated. After incubation for 2 h at 37°C, reaction mixtures were subjected to SDS gel electrophoresis and cleavage was measured as described in Fig. 2.

## Function of the protease activity of recA protein

The protease activity of recA protein clearly is responsible for the induction of temperate bacteriophages, and probably for induction of the *recA* gene itself. Thus it is required at least indirectly for recombinational repair of DNA, which occurs efficiently only if recA protein is amplified. Other *recA*-dependent processes such as mutagenesis might involve proteolytic modification of cellular proteins, as well as the strand pairing activity of recA protein.

Does the protease activity also have a direct role in genetic recombination? The fundamental reaction of recombination catalysed by purified recA protein on naked DNA *in vitro*

clearly does not utilise this activity<sup>8-11</sup>. Despite this, it is possible that the protease activity affects the rate of *recA* function *in vivo*. Cellular DNA is complexed with a variety of proteins, so that another role of the protease activity could be to remove such proteins to facilitate the exploration of duplex DNA that must precede strand pairing. An analysis of mutations that affect different activities of recA protein might indicate if this is true.

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# Morphogenetic genes *C* and *Nu3* overlap in bacteriophage $\lambda$

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*In bacteriophage  $\lambda$ , genes C and Nu3, two of the four cistrons which are essential for normal prohead formation, have overlapping nucleotide sequences. These genes are translated in the same reading frame so that the Nu3 protein is identical to the COOH-terminal one-third of the C protein. This structural relationship may provide for the functional interaction of the C and Nu3 proteins through their regions of structural homology during prohead assembly. The in-phase overlapping organisation of genes may constitute a general strategy to facilitate the mutual interaction of a pair of proteins through their common structural domains.*

MORPHOGENESIS of the head of bacteriophage  $\lambda$  is controlled by ten genes clustered in the left arm of the phage chromosome (Fig. 1a)<sup>1-7</sup>. Of these genes only *B*, *C*, *Nu3* and *E*, encoding proteins of molecular weights (MWs) 60,000, 58,000, 19,000 and 38,000, respectively, are essential for the production of the head precursor known as the prohead<sup>8-15</sup>. pNu3 acts as the scaffolding protein in prohead construction, directing the assembly of the major capsid protein, pE (refs 14-17). Nu3

protein is subsequently degraded and lost from the prohead structure<sup>14,15,17</sup>. pC is thought to be involved in the early stages of prohead assembly. It is ultimately fused to a minor fraction of the pE molecules in the prohead and cleaved to generate X1 and X2, two polypeptides differing only in the size of their pC-derived moiety. These two proteins, as well as pB and its cleavage product pB\*, are the minor components of the mature prohead<sup>14-19</sup>.

There is only one available amber mutation in gene *Nu3*—*ama8* (ref. 4). Previous examination of the proteins synthesised after infection of non-permissive *Escherichia coli* by  $\lambda$ Nu3<sub>ama8</sub> suggested an unusual relationship between pNu3 and pC (ref. 17). In these studies the mutation in gene *Nu3* resulted not only in the disappearance of pNu3 and the appearance of a Nu3 amber polypeptide, but also in the appearance of a lower MW form of pC. As C protein is known to be susceptible to cleavage<sup>17,18</sup>, the production of this altered form of pC was initially attributed to a rapid aberrant cleavage of pC in the absence of functional pNu3 (ref. 17). However, it was noted that the difference in MW between pNu3 and its amber peptide was very similar to the difference in MW between pC and the lower MW form of pC. An alternative explanation for these observations—that the gene products of *C* and *Nu3* have a common COOH terminus—was therefore considered. By this model the



two proteins are translated in the same reading frame and terminated at the same site. Thus, the amber mutation in gene *Nu3* is also an amber mutation in gene *C* causing premature termination in the translation of both proteins so that the pC and pNu3 amber polypeptides synthesised by  $\lambda$ Nu3<sub>ama8</sub>-infected cells differ in size from the respective wild-type polypeptides by the same amount. This model predicts that mutations within the promoter-proximal region of the *C* gene will affect only the *C* polypeptide, whereas mutations within the overlapping promoter-distal region will affect both pC and pNu3. Further, the Nu3 protein will be identical to the COOH-terminal region of the *C* protein, and tryptic peptides of pNu3 (except perhaps the NH<sub>2</sub>-terminal peptide) should be a subset of those derived from pC. In this article we describe experiments the result of which support the predictions of such a model.

### A missense mutation in gene *Nu3* does not alter the MW of the product of gene *C*

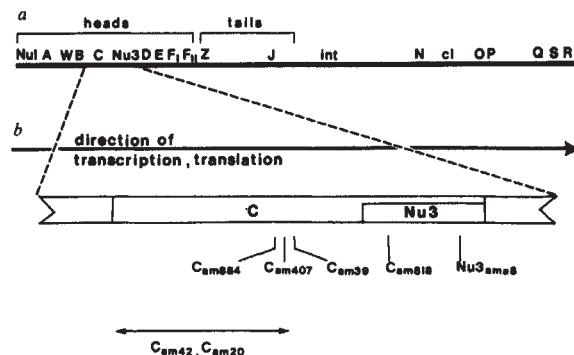
As a preliminary step in the investigation of the relationship between pC and pNu3, we compared the MWs of pC and pNu3 synthesised after infection of *E. coli* with either a  $\lambda$ Nu3 amber mutant or a  $\lambda$ Nu3 missense mutant. As originally observed by Ray and Murialdo<sup>17</sup>, infection of non-permissive cells with  $\lambda$ Nu3<sub>ama8</sub> results in the synthesis of two altered proteins, as seen in SDS-polyacrylamide gels: (1) pNu3 with a MW of 19,000 (ref. 17) is replaced by its amber fragment pNu3<sub>as</sub> of approximate MW 15,000, and (2) pC with a MW of 58,000 (ref. 20) is replaced by pC<sub>as</sub>(pC\* of Ray and Murialdo), with an apparent MW of about 54,000 (Fig. 2a). If the production of pC<sub>as</sub> by  $\lambda$ Nu3<sub>ama8</sub> were the result of rapid cleavage of a wild-type *C* protein in the absence of functional pNu3, as previously suggested<sup>17</sup>, the *C* protein made after infection of cells with a different mutant in gene *Nu3* might suffer similar cleavage and be present as a fragment with the same MW as pC<sub>as</sub>. This is not the case.  $\lambda$ Nu3<sub>78</sub>, an absolute defective mutant in gene *Nu3* (ref. 5), directs the synthesis of a pC with a MW of 58,000 the same mobility as that produced in cells infected with  $\lambda$ C<sub>I</sub><sub>u</sub> (wild-type  $\lambda$ ) (Fig. 2a, d). Thus, a nonsense mutation, but not a missense mutation in gene *Nu3*, can alter the size of pC. This result favours the model in which *C* and *Nu3* are overlapping genes.

### A gene *C* mutant is also a gene *Nu3* mutant

To test the prediction that mutants in the promoter-distal one-third of the *C* cistron will also be mutant in the *Nu3* cistron, we examined the proteins synthesised in non-permissive *E. coli* infected with a series of amber mutants in gene *C* (Fig. 2a). For some of these infections, the *C* amber polypeptide can be resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as indicated in Fig. 2, and its size can be used to estimate the position of the corresponding amber mutation within the *C* gene. Assuming that genes *C* and *Nu3* terminate at the same site, a map of the *C*-*Nu3* region of the  $\lambda$  chromosome can be constructed from the MWs of pC and pNu3, and from the MWs of the amber fragments observed (Fig. 1b). When this is done, five of the six *C* mutants tested fall into order within the amino-terminal region exclusive to *C*, whereas Cam818 falls into the region shared by *C* and *Nu3*. As expected, the mutants located in the non-overlapping portion of the *C* gene fail to produce *C* protein but make normal amounts of Nu3 protein, whereas  $\lambda$ C<sub>am818</sub> makes neither pC nor pNu3. This scheme agrees with genetic data which show that  $\lambda$ C<sub>am818</sub> is the only *C* mutant of this set that fails to complement  $\lambda$ Nu3<sub>ama8</sub> (data not shown).

### The electrophoretic mobilities of both pC and pNu3 are altered by insertion of tyrosine at position am818

The above data are consistent with the overlapping genes model but do not exclude the possibility of double mutations in the strains that affect both pC and pNu3, or polarity of the C<sub>am818</sub>

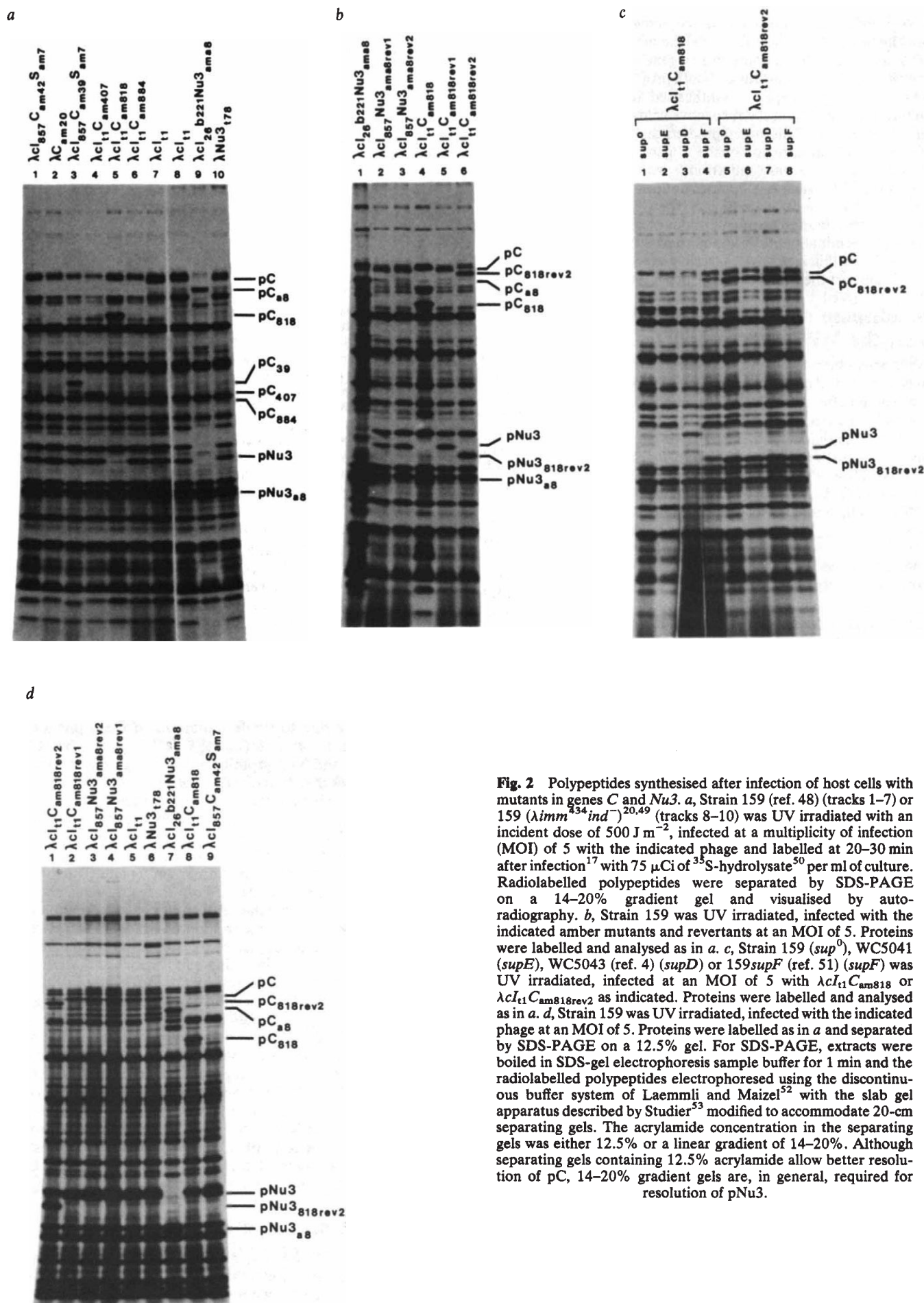


**Fig. 1** Genetic map of  $\lambda$  and expanded map of the *C*-*Nu3* region. *a*, The vegetative map of  $\lambda$  (not to scale) showing the relative positions of the morphogenetic genes involved in head formation, *Nu1*-*F<sub>III</sub>*, and tail assembly, *Z*-*J*. *b*, An expanded map of the *C*-*Nu3* region drawn to scale. Positions of the *C* and *Nu3* genes and of amber sites are determined from the apparent MWs of the corresponding polypeptides and amber fragments as shown in Fig. 2a. The relative positions of the mutations are the same as determined genetically. The  $\lambda$ C<sub>am20</sub> and  $\lambda$ C<sub>am42</sub> mutants, whose amber polypeptides were not observed, have been mapped genetically<sup>47</sup>, and their amber sites lie to the left of Cam39. The direction of transcription and translation is as indicated.

mutation on gene *Nu3*. To confirm that the observed effects are the result of single mutations, spontaneous revertants of  $\lambda$ C<sub>I</sub><sub>u</sub>Nu3<sub>ama8</sub> and  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub> were selected on strain 594, a non-suppressing host. Revertants were obtained at frequencies typical for single mutations ( $10^{-7}$ – $2 \times 10^{-7}$ ) and two revertants of each parent were selected for further study. The polypeptides synthesised in non-suppressing hosts infected with these revertants are shown in Fig. 2b and d. The two revertants of  $\lambda$ C<sub>I</sub><sub>u</sub>Nu3<sub>ama8</sub> and one revertant of  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub> (rev 1) produce pNu3 and pC of wild-type MW, indicating that the observed alterations of pNu3 and pC, in both  $\lambda$ C<sub>I</sub><sub>u</sub>Nu3<sub>ama8</sub> and  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub>, are due to single mutations in these phages. The other revertant of  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub>,  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub>rev2, directs the synthesis of *C* and *Nu3* proteins each having an apparent MW about 1,000 less than that of wild-type pC and pNu3. This result is another example of a simultaneous and identical alteration in the mobilities of these two proteins due to a single mutational event. Surprisingly, this mobility change of pC and pNu3 in  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub>rev2-infected cells may be due to the particular amino acid that is now inserted at the former amber site. The following experiment was therefore carried out to see if such a change in mobility could arise from the substitution of a single amino acid. Cells carrying different amber suppressors were infected with  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub> and the mobilities of pC and pNu3 were examined. Infection of *supD* (Ser-inserting suppressor) and *supE* (Gln-inserting suppressor) cells results in the synthesis of small amounts of pC and pNu3 of wild-type size (and also the amber fragment of *C*, Fig. 2c). *supF*-infected cells (Tyr-inserting), on the other hand, synthesise *C* and *Nu3* polypeptides having the same mobilities as those found in  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub>rev2-infected cells, that is, polypeptides with an apparent reduction in MW of 1,000. We conclude that it is the insertion of tyrosine at the C<sub>am818</sub> site that is responsible for the altered mobilities of these polypeptides. The MWs of the *C* and *Nu3* proteins remain unchanged in infections of all three suppressor strains with  $\lambda$ C<sub>I</sub><sub>u</sub>C<sub>am818</sub>rev2. It seems likely that in  $\lambda$ C<sub>am818</sub>rev2 the amber codon has been changed to code for Tyr.

### The Nu3<sub>78</sub> mutation alters the isoelectric points of both pC and pNu3

To probe further the model that the *C* and *Nu3* proteins share a common coding region, we investigated the properties of these two proteins synthesised by  $\lambda$ Nu3<sub>78</sub>-infected cells.  $\lambda$ Nu3<sub>78</sub>, as





mentioned above, is an absolute defective mutant in gene *Nu3* that produces C and Nu3 proteins of wild-type MW. Extracts of cells infected with this mutant were analysed by two-dimensional isoelectric focusing and SDS-PAGE<sup>21</sup>. Comparison of the two-dimensional pattern of polypeptides from  $\lambda$ CI<sub>11</sub>- and  $\lambda$ Nu3<sub>178</sub>-infected hosts reveals that both pNu3<sub>178</sub> and pC<sub>178</sub> are more basic than the respective wild-type proteins (Fig. 3). This is most clearly seen when samples from extracts of  $\lambda$ CI<sub>11</sub>- and  $\lambda$ Nu3<sub>178</sub>-infected cells are mixed and run together in the same gel (Fig. 3c). All other proteins synthesised in the mutant infection have the same mobilities as in a wild-type infection, with the exception of three minor proteins of slightly lower MW than pC which are polypeptides derived from C protein (pC-associated minor bands of Ray and Murialdo)<sup>17</sup>.

### The tryptic peptides of pNu3 are a subset of the tryptic peptides of pC

<sup>35</sup>S-labelled proteins C and Nu3 and polypeptides pC<sub>as</sub> and pNu3<sub>as</sub> were isolated by SDS-PAGE, eluted from the gel, digested with trypsin and the resulting peptides separated by electrophoresis and chromatography on cellulose thin layer plates<sup>22</sup>. The radiolabelled tryptic peptides of pNu3 are all contained within the peptide map of pC (Fig. 4), indicating that these two proteins have common amino acid sequences. In addition, the tryptic peptide missing (peptide 1) from the fingerprint of the amber polypeptide pNu3<sub>as</sub> is also absent in the peptide map of pC<sub>as</sub>. This indicates that the *Nu3*<sub>amas</sub> mutation results in the production of amber polypeptides of C and Nu3 both lacking the same COOH-terminal amino acid sequence, and demonstrates that the Nu3 protein and the COOH-terminal portion of the C protein are encoded by the same DNA.

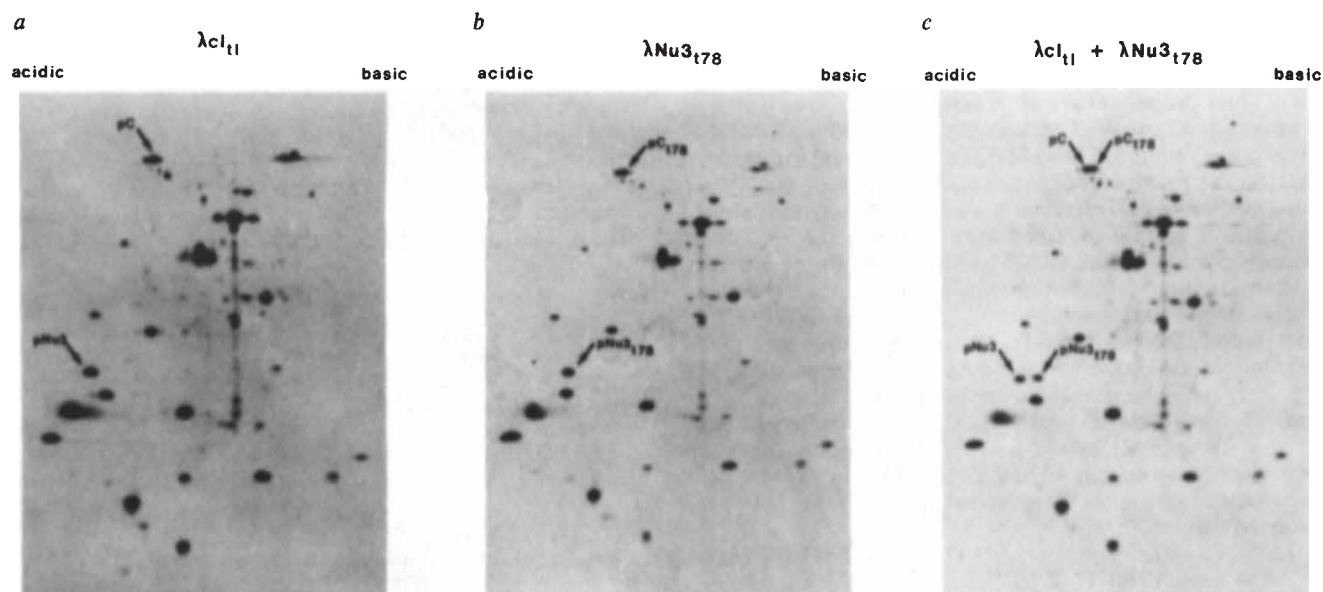
### Conclusions and implications

The results presented here demonstrate that the Nu3 protein and the COOH-terminal one-third of the C protein are encoded on the same DNA. The Nu3 protein is not derived from pC by proteolytic processing because all the C amber mutants located in the NH<sub>2</sub>-terminal (non-overlapping) portion of the gene direct the synthesis of normal amounts of Nu3 protein. It is possible that C protein is not a primary gene product but arises from the fusion of Nu3 protein with a polypeptide encoded in the region immediately preceding gene *Nu3*. However, this

seems unlikely as co-infection of cells with C amber and *Nu3* amber mutants does not result in the production of pC with wild-type MW. Furthermore, pulse-chase experiments fail to provide any evidence of a short-lived precursor to pC (data not shown). We therefore believe that the *Nu3* gene lies within the C gene. Translation of the two proteins is in the same reading frame and termination of translation occurs at or near the same site.

It is not known whether translation of the Nu3 protein is initiated internally to C on a common mRNA or whether the two proteins are translated separately from two different mRNAs. In two other instances of overlapping genes in which this has been investigated—the polyoma proteins VP2 and VP3 (refs 23, 24), and A and A\* proteins of  $\Phi$ X174 (ref. 25)—all the proteins seem to be translated from distinct species of mRNA. In the case of the C and Nu3 proteins of  $\lambda$ , there is no evidence to exclude the possibility of translation from either a common mRNA or separate RNAs. Although there is probably not a separate promoter for the transcription of the *Nu3* gene<sup>26</sup>, it is possible that the primary polycistronic late transcript initiated from *P<sub>L</sub>* (ref. 27) is modified so that pC and pNu3 are translated from different species of mRNA. On the other hand, the functional half-life of the C mRNA is about half that of the *Nu3* mRNA<sup>28</sup>, which is compatible with their being translated from the same transcript. Also, about two- to threefold more copies of pNu3 than pC are synthesised in an infected cell (data not shown), suggesting that there is a relatively strong ribosome binding site for *Nu3* within the C gene. This might allow internal initiation for the translation of pNu3 on a common transcript. In any event, we have observed that translation of the C protein probably interferes with translation of pNu3. In cells infected with  $\lambda$ C<sub>am737</sub>, the most promoter-proximal amber mutation in the C gene, the amount of Nu3 protein synthesised is about 1.5–2 times as much as is produced in a wild-type infection (data not shown). Although probably not important in the case of the C and Nu3 proteins of  $\lambda$ , interference in the synthesis of the promoter-distal protein by the translation of the promoter-proximal polypeptide could be a means of regulating the level of the downstream protein in an overlapping gene pair<sup>29–32</sup>.

There are several examples of overlapping genes in the small double-stranded bacteriophages  $\Phi$ X174 (refs 32–35) and G4 (refs 36, 37) in the RNA phage  $\phi$ Q (refs 38–40) and in the

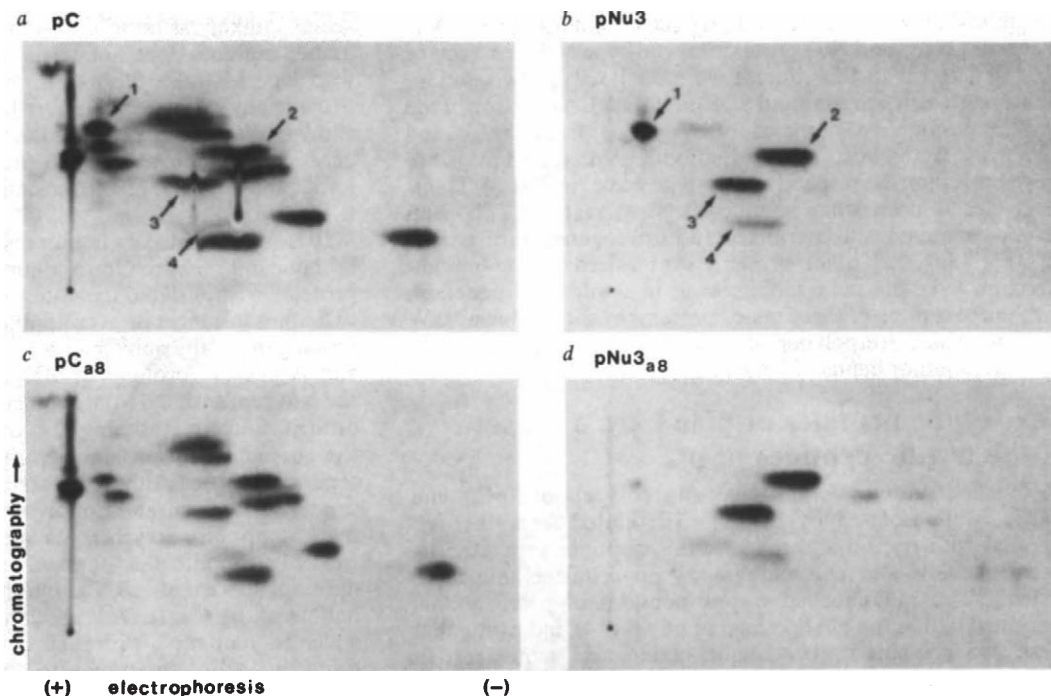


**Fig. 3** Two-dimensional separation of proteins synthesised in  $\lambda$ Nu3<sub>178</sub>-infected cells. Strain 159 ( $\lambda$ imm<sup>434</sup>ind<sup>-</sup>) was UV irradiated, infected with either  $\lambda$ CI<sub>11</sub> or  $\lambda$ Nu3<sub>178</sub> at an MOI of 5, and labelled with <sup>35</sup>S-hydrolysate at 20–30 min after infection. The proteins were separated in the first dimension by isoelectric focussing in gels containing 2% ampholytes (w/v) (20% pH 3.5–10, 80% pH 5–8) and in the second dimension by SDS-PAGE on a 14–20% gradient gel<sup>21</sup>. Radiolabelled polypeptides were visualised by fluorography<sup>54</sup>.

a,  $\lambda$ CI<sub>11</sub>; b,  $\lambda$ Nu3<sub>178</sub>; c, 1:1 (v/v) mixture of samples in a and b.

**Fig. 4** Tryptic fingerprints of pC, pNu3, pC<sub>a8</sub> and pNu3<sub>a8</sub>. Strain 159 was UV irradiated and infected with either  $\lambda$ CI<sub>26</sub>b221E<sub>am815</sub> for preparation of pC and pNu3 or  $\lambda$ CI<sub>857</sub>Nu3<sub>ama8</sub>FI<sub>am785</sub> for preparation of pC<sub>a8</sub> and pNu3<sub>a8</sub>. Use of these particular phage strains allows isolation of pNu3 and pNu3<sub>a8</sub> relatively free of contaminating labelled phage proteins. The infected cells were labelled at 20–30 min after infection with <sup>35</sup>S-hydrolysate and proteins were separated by SDS-PAGE on a 12.5% gel. Radiolabelled polypeptides were visualised by autoradiography and pC, pNu3, pC<sub>a8</sub> and pNu3<sub>a8</sub> were cut out of the gel, eluted, oxidised with performic acid, and digested with trypsin<sup>22</sup>.

Analysis of the isolated but undigested proteins by SDS-PAGE on a 14–20% gradient gel indicated that pC, pNu3 and pC<sub>a8</sub> were free of contaminating labelled proteins. pNu3<sub>a8</sub> contained minor amounts of contaminating material. The tryptic peptides were separated by electrophoresis and chromatography on cellulose thin layer plates<sup>22</sup>. Peptides from pC(a) and pNu3 (b) were run simultaneously, in parallel, on one thin layer plate, as were peptides from pC<sub>a8</sub> (c) and pNu3<sub>a8</sub> (d). Peptides derived from pC and pNu3 having identical mobilities are indicated by arrows.



papovaviruses polyoma<sup>41</sup> and SV40 (refs 42, 43). It has been argued that the overlapping organisation of genes in these viruses offers the advantage of efficient use of a limited coding capacity. From this viewpoint, the discovery of an overlapping pair of genes in  $\lambda$  may be somewhat surprising, as  $\lambda$  has a relatively large genome with a large non-essential region which itself may contain an overlapping gene pair (C. Epp and M. Pearson, personal communication). This may indicate that an overlapping genetic arrangement is a fairly widespread phenomenon with additional advantages to the organism. The fact that pC and pNu3 are translated in the same reading frame suggests that the role of the overlap, in this instance, is to generate a pair of proteins with a specific structural relationship essential for proper prohead assembly. The overlapping arrangement of *C* and *Nu3* ensures that pNu3 and the COOH-terminal one-third of pC have identical amino acid sequences and are therefore structurally very similar, whereas the NH<sub>2</sub>-terminal portion of pC is structurally unique. A possible model for  $\lambda$  prohead morphogenesis is one in which the common domains of pNu3 and the COOH-terminal region of pC co-polymerise during the formation of a prohead core structure. Molecules of pC thus assembled into the core could further interact through their unique NH<sub>2</sub>-terminal domains with other morphopoietic proteins such as pE, during the polymerisation of the capsid. (An NH<sub>2</sub>-terminal portion of pC is known to interact with pE, the major capsid protein, and by fusion with pE and cleavage of the pC–pE hybrid polypeptide produces the two minor prohead components X1 and X2 (ref. 15).) In this way, pC might be viewed as a linker between two different substructures, the prohead core and coat.

Of the overlapping genes translated in phase that have been described to date (*A* and *A\** of  $\Phi$ X174 and G4, *VP2* and *VP3* of the papovaviruses, and *A*<sub>1</sub> and *coat* of *Q $\beta$* ), the *VP2* and *VP3* proteins of polyoma and SV40, and the *A*<sub>1</sub> and *coat* proteins of *Q $\beta$*  (ref. 44) are essential for viral morphogenesis. The *VP2* and *VP3* proteins, organised genetically in an analogous fashion to pC and pNu3 of  $\lambda$ , have common COOH-terminal regions whereas the coat protein and *A*<sub>1</sub> protein, a readthrough product

of the *coat* gene, have identical NH<sub>2</sub>-terminal sequences. In polyoma, recent experiments suggest that *VP2* and *VP3* form the pentons of the capsid<sup>45</sup>. This is consistent with the model that these proteins interact with one another because of their common amino acid sequence. Thus, the in-phase overlapping arrangement of genes could be a general strategy to generate a pair of proteins, such as pC and pNu3, with regions of structural homology and non-homology. The structural relationship between the proteins of such a pair may result in their mutual interaction through the shared structural domain<sup>46</sup> while allowing individual specialised reactions through their unique structural domains. In this way, interactions involving each protein of the pair could be physically coupled to facilitate the assembly of a complex biological structure.

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# Amino acid sequence of homogeneous antibodies to dextran and DNA rearrangements in heavy chain V-region gene segments

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*The complete variable region sequences from ten antibodies and two myeloma proteins binding  $\alpha$ -1,3 dextran have been determined. The diversity patterns of these homogeneous antibody molecules suggest that the variable regions of heavy chains are encoded by separate variable (V) and joining (J) gene segments. The most striking feature of these data is the extensive sequence variability of a region that we denote the D (diversity) segment which is located at the junction between the V and J segments in the centre of the third hypervariable region. The D segment diversity may arise from a novel somatic mutational mechanism or may be encoded by multiple D gene segments. For the first time, the amino acid sequence correlates of several V region idiotypes are determined.*

STUDIES of immunoglobins at the protein and DNA levels have led to striking advances in our understanding of the organisation and evolution of antibody genes and mechanisms of antibody diversity<sup>1-4</sup>. There are three unlinked families or clusters of antibody genes—the  $\lambda$  and  $\kappa$  gene families encode light (L) chains and the heavy (H) gene family encodes heavy chains<sup>5</sup>. The  $\kappa$  or  $\lambda$  gene is composed of four distinct gene segments, leader (L), variable (V), joining (J) and constant (C), which are separated by intervening sequences in undifferentiated or germ-line DNA<sup>6-12</sup>. Heavy chain genes probably have a similar organisation of gene segments (P. Early, H. Huang, M. Davis and L.H., unpublished observations). During the differentiation of antibody-producing cells, the V<sub>L</sub> and J<sub>L</sub> gene segments are juxtaposed and together encode the variable or antigen-binding domain of the antibody molecule. (The term V or J segment will refer to the corresponding protein sequence; the term V or J gene segment will refer to the corresponding DNA coding regions.) Comparative amino acid sequence analyses of V regions showed that for both light and heavy chains, there are three regions of extreme variability termed hypervariable regions<sup>13,14</sup>, which fold in three dimensions to constitute the walls of the antigen-binding site<sup>15,16</sup>. The diversity in the hypervariable regions of L chains has been most extensively studied and explained by a variety of genetic mechanisms. (1) There are

many germ-line V <sub>$\kappa$</sub>  (and J <sub>$\kappa$</sub> ) gene segments<sup>5,10</sup>. (2) A single V <sub>$\kappa$</sub>  gene segment may be joined to many different J <sub>$\kappa$</sub>  gene segments and conversely, a single J <sub>$\kappa$</sub>  gene segment may be joined to many different V <sub>$\kappa$</sub>  gene segments<sup>17</sup>. As the boundary of the V<sub>L</sub> and J<sub>L</sub> segments falls within the third hypervariable region, the combinatorial joining of these segments contributes to the diversity of antigen-binding sites. (3) Random single base mutations may occur throughout the V<sub>L</sub> (and J<sub>L</sub>) gene segments and those variants exhibiting altered and useful antigen-binding properties may be selected for clonal expansion. For example, 12 of 18 V <sub>$\lambda$</sub>  regions examined were identical to one another<sup>18-20</sup>. The six variant V <sub>$\lambda$</sub>  sequences differed from the 12 identical sequences by between one and three amino acid substitutions. All these substitutions occur in the hypervariable regions. These data suggest that a single V <sub>$\lambda$</sub>  gene is often expressed unchanged and less frequently is modified by somatic mutation to produce the variants. Recent DNA sequence analyses of germ-line and myeloma  $\lambda$  gene segments are consistent with this supposition<sup>6,11,12</sup>. The pattern of diversity wherein variant V region sequences exhibit few substitutions mostly concentrated in the hypervariable regions will be denoted  $\lambda$ -type diversity. (4) A site-specific recombinational mechanism has been postulated to explain amino acid sequence diversity at the junction of V <sub>$\kappa$</sub>  and J <sub>$\kappa$</sub>  gene segments<sup>21-23</sup>. This diversity is reflected as single codon substitutions at the N-terminal residue of the J <sub>$\kappa$</sub>  segment and as the insertion or deletion of single codons precisely at the V-J junction<sup>23</sup>. This pattern of diversity will be denoted the V-J junctional diversity.

We have analysed the diversity patterns in mouse antibodies directed against a simple antigenic-determinant,  $\alpha$ -1,3 dextran, in order to determine the relative contributions of the diversification mechanisms discussed above. The hybridoma technique of Köhler and Milstein facilitates the analyses of antibody diversity by making large quantities of antigen-induced, homogeneous antibody molecules available<sup>24</sup>. This is accomplished by fusing normal antibody-secreting cells with mouse plasmacytoma cells<sup>25</sup>. The hybrid cells generate immortal cell lines that secrete large amounts of homogeneous antibody molecules of the two parental cell types. The light chains of the dextran antibodies are all of the  $\lambda$  type and appear to be very similar, if not identical, to one another. Hence, most of the diversity in the dextran system appears to reside in the

**Table 1** RNA codons of positions 100 and 101 from V<sub>H</sub> regions of myeloma and hybridoma proteins binding dextran\*

| V-J segments†                  | Proteins | Codons‡      |              |
|--------------------------------|----------|--------------|--------------|
|                                |          | 100          | 101          |
| V <sub>1</sub> -J <sub>1</sub> | M104E    | a b c<br>UAY | a b c<br>GAY |
|                                | J558     | AGZ<br>CGX   | UAY          |
|                                | Hdex2    | AAZ          | UAY          |
|                                | Hdex3    | AGZ<br>CGX   | GAY          |
|                                | Hdex7    | GAX          | GAY          |
|                                | Hdex6    | AGY<br>UCX   | CAY          |
| V <sub>2</sub> -J <sub>1</sub> | Hdex8    | UAY          | GAY          |
| V <sub>3</sub> -J <sub>1</sub> | Hdex9    | AGZ<br>CGX   | UAY          |
| V <sub>4</sub> -J <sub>1</sub> | Hdex10   | GUX          | AAZ          |
| V <sub>1</sub> -J <sub>2</sub> | Hdex4    | AAZ          | GAY          |
|                                | Hdex5    | AGY<br>UCX   | AAZ          |
| V <sub>1</sub> -J <sub>3</sub> | Hdex1    | AAZ          | UAY          |

\* These codon assignments are derived from the corresponding amino acid sequences

† The V and J segments are defined in Fig. 2.

‡ Y denotes pyrimidine; Z designates purine; and X denotes any one of the four bases.

heavy chains. We report here the complete amino acid sequences from the variable regions of 10 heavy chains derived from hybridoma antibodies to dextran. These data suggest that heavy chains have a D (diversity) segment located in the heart of the third hypervariable region. The D segment may be encoded by a separate germ-line gene segment or generated by a new type of somatic mutational mechanism.

## Immunoglobulins binding dextran

Immunoglobulins binding dextran exhibit limited heterogeneity in several respects. First, preliminary amino acid sequence studies on two myeloma proteins binding  $\alpha$ -1,3 dextran, M104E and J558, revealed that the V<sub>L</sub> regions are identical<sup>18,19</sup> and that the V<sub>H</sub> regions are identical through their first hypervariable regions<sup>26</sup>. The N-terminal sequences of V<sub>H</sub> regions derived from normally-induced pooled serum antibodies to  $\alpha$ -1,3 dextran also are identical to their myeloma counterparts<sup>27</sup>. Second, isoelectric focusing analyses of anti-dextran antibodies of the IgG class demonstrate limited heterogeneity with considerable sharing of bands between sera from different mice<sup>28</sup>. Moreover, the serum antibody light chains are identical to one another and to their myeloma counterparts by isoelectric focusing criteria<sup>27</sup>. Third, the light chains from the 10 hybridomas and 2 myeloma proteins reported here are all of the  $\lambda$  type and have identical isoelectric focusing patterns (J.S., unpublished observation). Thus, light chains from dextran antibodies appear to be very similar, if not identical, to one another. Therefore, most of the diversity in the antibodies to  $\alpha$ -1,3 dextran must reside in the heavy chains. Fourth, the anti- $\alpha$ -1,3 dextran antibodies can be divided into several general categories by idiotype analyses<sup>29</sup>. More than half of the anti- $\alpha$ -1,3 dextran antibodies from mice immunised with the branched dextran B1355 share a variable-region antigenic determinant or idiotype with both the M104E and J558 proteins. This idiotype is absent from non-dextran binding immunoglobulins and  $\lambda$  light chains and is denoted the 'cross-reactive' or IdX idiotype. Antibodies with the IdX idiotype are synthesised by multiple different antibody-producing clones. Additional idiotypes specific for either the M104E or the J558 immunoglobulins are present on a minor fraction of the

normally-induced antibodies. These idiotypes are denoted as individual or IdI idiotypes (for example, IdIM104E and IdIJ558). Another general category of dextran antibodies is that of those lacking the IdX determinant (IdX negative). The IdX-positive and IdX-negative antibodies to dextran are structurally very similar in that their light chains are identical by isoelectric focusing analysis and the heavy chains from both the IdX-positive and IdX-negative pools of serum antibodies are identical to the heavy chains of the M104E and J558 immunoglobulins for their N-terminal 27 and 53 residues, respectively<sup>27</sup>. These observations show that much of the V domain diversity in normal antibodies to  $\alpha$ -1,3 dextran resides in the heavy chain beyond residue 53. Thus, we turned to the amino acid sequence analysis of homogeneous normal antibodies to  $\alpha$ -1,3 dextran.

## Diversity patterns in V<sub>H</sub> regions from antibodies binding dextran

Figure 1 gives the complete amino acid sequences of V<sub>H</sub> regions from 2 myeloma proteins and 10 hybridoma antibodies that bind dextran. The hypervariable regions and the variable region site of carbohydrate attachment are indicated. These V<sub>H</sub> regions are compared to the published sequence of the myeloma M104E V<sub>H</sub> region<sup>30</sup> with amino acid substitutions indicated by the one-letter code<sup>31</sup>.

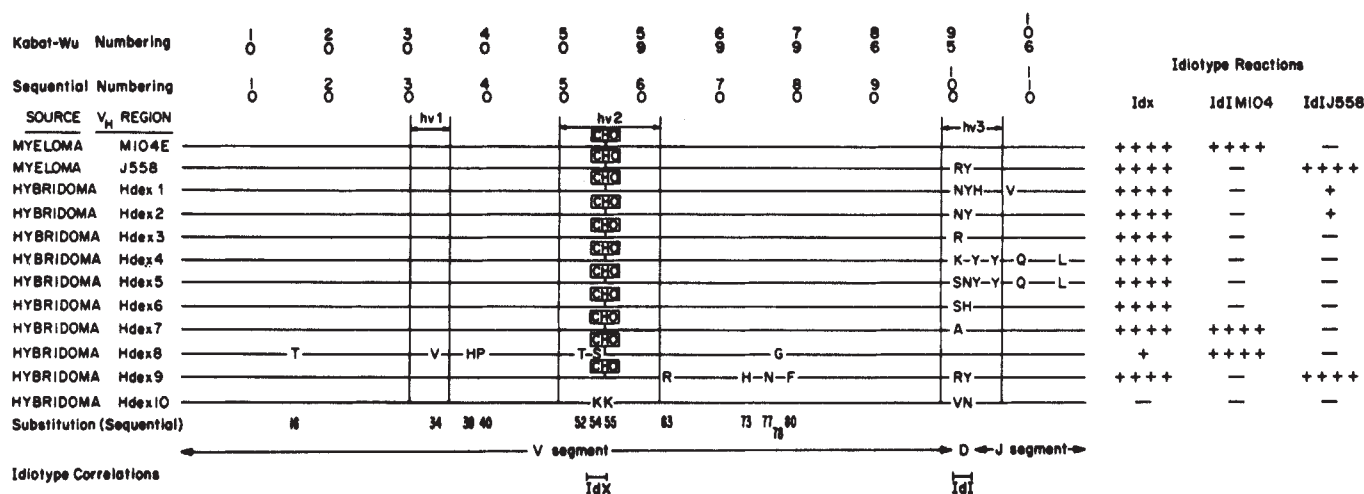
Two features are of interest in this set of V<sub>H</sub> regions. First, each of these 12 V<sub>H</sub> regions differs from all of the others by 1 to 13 amino acid substitutions (Fig. 1). We have not yet detected a pair of identical V<sub>H</sub> regions in 12 attempts. Accordingly, the V<sub>H</sub> sequence diversity in the antibody response to  $\alpha$ -1,3 dextran is extensive. Second, patterns of variability divide the V<sub>H</sub> regions from dextran antibodies into three distinct sections—V(1–99), V(100–101) and V(102–117). The highly variable section, V(100–101), separates the other two sections which are relatively conserved. Section V(100–101) exhibits nine different sequences in the 12 V<sub>H</sub> regions (Fig. 2). Indeed, position 100 has eight alternatives and position 101 has four. In contrast, nine V<sub>H</sub> regions have identical sequences for section V(1–99) and the remaining V<sub>H</sub> regions differ by between two and seven residues from the others. Likewise, nine V<sub>H</sub> regions have identical sequences for the section V(102–117), two have a second sequence (Hdex4 and Hdex5) and one has a third sequence (Hdex1). Thus, the relatively conserved sections V(1–99) and V(102–117) are separated by two highly variable residues at positions 100 and 101.

This same three-section pattern is seen when 20 completely sequenced V<sub>H</sub> regions are aligned by homology with their anti-dextran counterparts (Fig. 3). The section homologous to V(1–99) can clearly be defined in each of these 20 V<sub>H</sub> regions and each terminates precisely at the position homologous to 99 in the heavy chains binding dextran (Fig. 3). The homology relationships among the V(102–117) sections are also obvious. The V(102–117) sections from the various V<sub>H</sub> regions are identical in size, apart from the levan-binding immunoglobulins, and exhibit extensive amino acid sequence homology to one another (56–100% sequence identity). Accordingly, sections homologous to V(1–99) and V(102–117) in the dextran V<sub>H</sub> regions are clearly present in all of the other V<sub>H</sub> regions examined to date. In contrast, there is no homology in the V(100–101) regions between these two sections because of extensive sequence and size variability (Fig. 3). The sections analogous to V(100–101) can be defined only as those residues remaining after the V(1–99) and V(102–117) sections have been assigned for each V region by homology.

## Implications of V<sub>H</sub> diversity patterns for gene organisation

The patterns of amino acid diversity in the V<sub>H</sub> regions from antibodies binding  $\alpha$ -1,3 dextran allow us to define tentatively V<sub>H</sub> and J<sub>H</sub> segments with properties similar to their light chain counterparts (Figs 1–3). The V(1–99) section appears to correspond to the V<sub>H</sub> segment and V(102–117) section appears to





**Fig. 1** The amino acid sequences and idiotypes from V regions of myeloma proteins and homogeneous hybridoma antibodies binding dextran. The myeloma protein M104E is used as a prototype sequence and variation from this sequence is indicated. The hypervariable regions are indicated by hv1, hv2 and hv3, respectively. CHO indicates the attachment of a carbohydrate moiety. The one-letter amino acid code of Dayhoff is used<sup>31</sup>. The V and J segments are indicated by arrows. The D segment includes positions 100 and 101. Sequential numbering denotes consecutive numbering of the V<sub>H</sub> regions. The Kabat-Wu numbering is from ref. 36. Details of the complete heavy chain sequence of the M104E immunoglobulin are published elsewhere<sup>30</sup>. A similar strategy was used for the sequence analysis of the V<sub>H</sub> regions from the hybridomas. The positions of amino acid substitutions in V segments are indicated. Eight hybrids were derived from individual BALB/c mice: Hdex6, 7, 8 and 9 were obtained at 5 or 6 days after a single injection of 100 µg dextran B1355 in complete Freund's adjuvant (CFA), and Hdex2, 3, 4 and 10 were obtained 1-3 days after hyperimmunisation with dextran in CFA followed 1-2 months later with 3 interperitoneal injections of  $2 \times 10^9$  *Escherichia coli* at 2-day intervals. Hybrids Hdex1 and 5 were derived from a single (BAB-14 × BRVR)<sub>F1</sub> mouse at 7 days after the dextran-*E. coli* protocol. Hybrids were generated as described by Galfre *et al.*<sup>41</sup> using the HGPRT-deficient MPC-11 ( $\gamma_{2b}$ ) line, 45.6TG1.7, (Hdex1, 3, 4, 5 and 10) or a non-secreting variety of the MOPC-21 ( $\gamma_1\kappa$ ) line, NSI/1-Ag4-1, which synthesises but does not secrete  $\kappa$  chains (Hdex2, 6, 7, 8 and 9). Anti-dextran secreting hybrids were detected by direct binding of <sup>125</sup>I-dextran by culture supernates either in a tube-binding assay or after isoelectric focusing in polyacrylamide gels. Hybrids were cloned in soft agar<sup>42</sup> over feeder layers and grown in BALB/c mice as ascites tumours. The antibodies produced by the hybrids appeared representative of those found in the serum at the time of fusion. Idiotypes were determined as described by ref. 29. +++++ Indicates a reaction 10 times as strong as +; - indicates no reaction.

represent the J<sub>H</sub> segment. The V(100-101) section will be called the D (diversity) segment and its origin will be discussed.

The extensive variability in these V<sub>H</sub> regions at position 100 appears to mark the C-terminal end of the V<sub>H</sub> segment, just as extensive variability at position 96 marks the C-terminal end of the V segment in kappa light chains<sup>17</sup>. The correspondence of V(1-99) to a V<sub>H</sub> gene segment is confirmed by the DNA sequence analysis of a germ-line V<sub>H</sub> gene segment which ends precisely at codon 99 (P. Early, H. Huang, M. Davis and L.H., unpublished observation).

The J<sub>H</sub> segment includes at least positions 102-117. The C-terminal boundary of the J<sub>H</sub> segment at position 117 has been defined by the DNA sequence analysis of an expressed V<sub>H</sub>-J<sub>H</sub> gene (H. Huang, P. Early, M. Davis and L.H., unpublished observations). This analysis demonstrates that the C<sub>H</sub> region begins at a position homologous to residue 118 in the heavy chains from antibodies binding dextran, in agreement with the J<sub>H</sub> and C<sub>V1</sub> gene segment boundaries defined by DNA sequence analysis of a C<sub>V1</sub> gene<sup>32</sup>. The N-terminal boundary of the J<sub>H</sub> segment is unclear. Figure 2 shows three J<sub>H</sub> sequences which differ from each other by two or three two-base substitutions (J<sub>1</sub>, J<sub>2</sub> and J<sub>3</sub>). Position 102 is the first position in the J<sub>H</sub> segment at which linkage relationships among amino acid residues appear. A tryptophan at position 102 is invariably linked to valine, alanine and valine residues at positions 106, 109 and 113, respectively, in the J<sub>1</sub> segments, whereas the tyrosine at position 102 is linked to tyrosine, glutamine and leucine at these positions in the J<sub>2</sub> segments (Fig. 2). These linkage relationships imply that the J<sub>1</sub> and J<sub>2</sub> segments are encoded independently in the germ-line. The D segment comprises positions 100 and 101 and may or may not be a part of the J segment, depending on how V-J junctional diversity arises. These assignments of the V<sub>H</sub>, J<sub>H</sub> and D segment boundaries also are supported by the homology relationships exhibited among the other completely sequenced V<sub>H</sub> regions (Fig. 3).

The D and J<sub>H</sub> segments comprise almost the entire third hypervariable region (Fig. 2). This is in striking contrast to the J<sub>κ</sub> segment which just extends into the third hypervariable region by a single residue<sup>17</sup>. Moreover, the size variation seen in the D segment of heavy chains is far more extensive than that seen at the V-J boundary of kappa chains.

Rudikoff and coworkers have used more limited V<sub>H</sub> region data to suggest the V<sub>H</sub> segment extends from 1 to 98 and the J<sub>H</sub> segment from 105 to 117. They conclude that positions 99 to 104 may be part of the V segment or, alternatively, part of the J segment<sup>33</sup>. These assignments are different from those made here.

## Implications of V<sub>H</sub> diversity patterns for mechanisms of antibody diversity

The V<sub>H</sub> regions from dextran antibodies all differ from one another. Let us analyse possible contributions to this diversity by multiple germ-line gene segments, somatic mutation and the combinatorial joining of V<sub>H</sub> and J<sub>H</sub> segments.

**Germ-line V<sub>H</sub> and J<sub>H</sub> gene segments.** Four different amino acid sequences corresponding to the V<sub>H</sub> gene segment are present in the 12 antibodies binding dextran and these are denoted V<sub>1</sub> to V<sub>4</sub> in Figs 1 and 2. The V<sub>1</sub> segment is expressed in nine different immunoglobulins binding dextran and, accordingly, appears to be encoded by a germ-line gene. This supposition follows from the improbability of generating the same V<sub>H</sub> sequence many times by somatic mutation<sup>4</sup>. The V<sub>2</sub>, V<sub>3</sub> and V<sub>4</sub> segments differ from V<sub>1</sub> by seven, four and two residue substitutions, respectively. The V<sub>2</sub>, V<sub>3</sub> and V<sub>4</sub> segments may be encoded by distinct germ-line V<sub>H</sub> gene segments or these sequences may arise from one or more somatic mutational mechanisms. However, the diversity patterns among the V<sub>2</sub>, V<sub>3</sub> and V<sub>4</sub> segments do not resemble either of the two somatic mutational patterns described earlier for light chains, λ-type diversity and V<sub>κ</sub>-J<sub>κ</sub> junctional diversity.

| Kabat-Wu 94<br>Sequential 98    | 95  | 96  | 97  | 98  | 99  | 100 | 101 | 102 | 103 | 105 | 109 | 113 | 117 |     |     |     |     |     |     |     |
|---------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                                 | hv3 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|                                 | D   |     |     |     |     |     |     |     | J   |     |     |     |     |     |     |     |     |     |     |     |
| J558                            | ARG | ASP | ARG | TYR | TRP | TYR | PHE | ASP | VAL | TRP | GLY | ALA | GLY | THR | THR | VAL | THR | VAL | SER | SER |
| Hdex 9                          | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| M104E                           | --- | --- | TYR | ASP | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hdex 8                          | --- | --- | TYR | ASP | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hdex 1                          | --- | --- | ASN | --- | HIS | --- | --- | --- | --- | VAL | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hdex 2                          | --- | --- | ASN | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hdex 3                          | --- | --- | --- | ASP | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hdex 10                         | --- | --- | VAL | ASN | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hdex 6                          | --- | --- | SER | HIS | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hdex 7                          | --- | --- | ALA | ASP | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Hdex 5                          | --- | --- | SER | ASN | TYR | --- | --- | --- | --- | --- | GLN | --- | --- | --- | --- | LEU | --- | --- | --- | --- |
| Hdex 4                          | --- | --- | LYS | ASP | TYR | --- | --- | --- | --- | --- | GLN | --- | --- | --- | --- | LEU | --- | --- | --- | --- |
| Number of<br>alternatives       |     | 8   | 4   | 3   |     |     |     | 2   | 2   | 2   |     |     |     |     | 2   |     |     |     |     |     |
| Number of base<br>substitutions |     |     |     | 3   |     |     |     | 2   | 2   | 2   |     |     |     |     | 1   |     |     |     |     |     |

V-J segments

V<sub>1</sub>J<sub>1</sub>V<sub>3</sub>J<sub>1</sub>V<sub>1</sub>J<sub>1</sub>V<sub>2</sub>J<sub>1</sub>V<sub>1</sub>J<sub>3</sub>V<sub>1</sub>J<sub>1</sub>V<sub>1</sub>J<sub>1</sub>V<sub>4</sub>J<sub>1</sub>V<sub>1</sub>J<sub>1</sub>V<sub>1</sub>J<sub>1</sub>V<sub>1</sub>J<sub>2</sub>V<sub>1</sub>J<sub>2</sub>

Fig. 2 The C-terminal portions of V<sub>H</sub> regions from immunoglobulins binding dextran. See Fig. 1 legend. The number of alternative amino acids for each position at which variability exists are indicated. The number of base substitutions denotes the minimum number of nucleotide changes required to convert the most common codon at a particular position into the alternative codons at that position. The V<sub>1</sub> to V<sub>4</sub> and J<sub>1</sub> to J<sub>3</sub> segments are defined. The third hypervariable region is denoted by hv3. Three pairs of proteins with identical sequences at positions 100 and 101 are set apart from the rest. The V<sub>H</sub>, D and J<sub>H</sub> segments are defined in Fig. 1.

The three different J<sub>H</sub> segments differ from one another by two to five residue alternatives (Fig. 2). Moreover, most of these J<sub>H</sub> segment substitutions require two or even three base substitutions and repeats of two of these sequences have been seen. These observations strongly suggest that the three J<sub>H</sub> sequences are directly encoded by three germ-line J<sub>H</sub> gene segments. The J<sub>H</sub> diversity noted in Fig. 3 appears to be significantly greater than the corresponding J<sub>L</sub> diversity<sup>17</sup> and, accordingly, may be encoded by a larger number of germ-line J<sub>H</sub> gene segments.

**Combinational joining.** The combinational joining of V<sub>H</sub> and J<sub>H</sub> gene segments to encode many distinct V<sub>H</sub> regions provides yet another source of antibody diversity (Fig. 4). For example, identical J<sub>H</sub> segments may be associated with differing V<sub>H</sub> segments (such as M104E and Hdex8; J558 and Hdex9). Conversely, identical V<sub>H</sub> segments may be associated with different J<sub>H</sub> segments (for example, Hdex1, Hdex2 and Hdex4) (Fig. 1). Even more striking is the observation that the J<sub>1</sub> segment in V<sub>H</sub> regions from antibodies binding dextran also occurs in the very different V<sub>H</sub> regions from myeloma proteins binding phosphorylcholine (Fig. 3). Likewise, the same J<sub>H</sub> segment may be found in heavy chains binding phosphorylcholine and galactan (for example, M167 and T601). Thus, the combinational joining of J<sub>H</sub> and V<sub>H</sub> gene segments appears to be an important source of diversity in these dextran-binding antibodies.

**D segment diversity.** This most striking diversity pattern in these V<sub>H</sub> regions (Figs 2, 3). Diversity in the D segments may arise from one of three general mechanisms: (1) a novel somatic mutation mechanism possibly occurring as a result of the joining of V<sub>H</sub> and J<sub>H</sub> gene segments; (2) a large number of germ-line J<sub>H</sub> or V<sub>H</sub> gene segments which include the D segment; or (3) a third gene segment (D) encoding positions 100 and 101 which is joined with the V<sub>H</sub> and J<sub>H</sub> gene segments during the differentiation of an antibody-producing cell. Let us consider what constraints the diversity patterns of the V<sub>H</sub> regions impose on each of these models.

Several observations argue against many germ-line V<sub>H</sub> gene segments extending from codons 1 to 101. First, identical D segments may be associated with different V<sub>H</sub> segments—M104E-Hdex8 and J558-Hdex9 (Fig. 1). Thus, the D segments

appear to associate independently with V<sub>H</sub> segments. Second, the T15 V<sub>H</sub> gene segment terminates at codon 99 (P. Early, H. Huang and L.H., unpublished observation). Accordingly, the D segment cannot be encoded as part of the V<sub>H</sub> gene segment for the T15 V<sub>H</sub> gene segment (Fig. 3). Identical D segments may be associated with different J<sub>H</sub> segments—Hdex1-Hdex2 and X44-J539 (Fig. 3). A more striking example of this independent association of D and J<sub>H</sub> segments is the observation that a J<sub>1</sub> segment is associated with very different D segments in immunoglobulins binding distinct haptens—T15 and M104E (Fig. 3). The independent segregation of D segments with both V<sub>H</sub> and J<sub>H</sub> segments suggests that the D coding regions are not merely germ-line extensions of V<sub>H</sub> or J<sub>H</sub> gene segments. In addition, homology comparisons among the V<sub>H</sub> regions very clearly define the V<sub>H</sub> and J<sub>H</sub> segments (Fig. 3). In contrast, the D segments lack constancy both in size (0 to 7 residues) and amino acid sequence (20 different sequences in 25 different V<sub>H</sub> regions compared) (Fig. 3). The V<sub>H</sub> regions in Fig. 3 would require a very large number of germ-line gene segments if the V<sub>H</sub> or J<sub>H</sub> gene segments include the D segment.

The D segment diversity is quite distinct from the V-J junctional diversity of kappa chains in several regards. First, there are extensive size differences among the D segments whereas the κ junctional diversity shows at most the insertion or deletion of just a single codon<sup>23</sup>. Second, the two codons for the D segment associated with a particular V<sub>H</sub>(V<sub>1</sub>) and a particular J<sub>H</sub>(J<sub>1</sub>) segment exhibit three different nucleotide alternatives at three positions—100a, 100b and 101a (Table 1). This observation is important because it suggests that D segment diversity cannot be generated solely by recombination between the V<sub>1</sub> and J<sub>1</sub> gene segments as is suggested by the site-specific recombinational model of mouse kappa chains<sup>21-23</sup>. This model allows for only two alternatives at any nucleotide position. Accordingly, the extensive variability, the size differences and the presence of three bases at several nucleotide positions in the D segments constitute a distinctly new pattern of diversity that must be explained by a novel mechanism for somatic mutation or the presence of multiple D gene segments.

The striking base invariance at nucleotide positions 101b and



101c in the midst of the remarkable adjacent diversity raises several interesting possibilities (Table 1). Perhaps these invariant nucleotides form part of a recognition site for an enzyme that might mediate somatic mutation or join the D and J<sub>H</sub> gene segments together. Alternatively, perhaps these invariant nucleotides are a part of the J<sub>H</sub> gene segment.

If the D segment is encoded by a gene segment separate from the V<sub>H</sub> and J<sub>H</sub> gene segments, then heavy chain genes differ in a major organisational feature from light chain genes. Moreover, the presence of a putative D gene segment creates a second gene segment boundary (V<sub>H</sub>-D and D-J<sub>H</sub>) at which junctional diversity of the type noted in kappa chains can occur<sup>21-23</sup>.

### Antigen-binding diversity and V<sub>H</sub> variability

The third hypervariable region forms an important part of the antigen-binding site<sup>15,16</sup>. Diversity in the dextran antibodies is concentrated in the third hypervariable region of heavy chains which includes the D segment and much of the J<sub>H</sub> segment (Fig. 2). Studies on the binding specificities of the myeloma proteins M104E and J558 which differ only in the D segment have demonstrated that the antigen-binding properties of these two molecules are quite distinct<sup>34,35</sup>. Accordingly, the mechanism for producing D segment diversity does have an important role in modifying the antigen-binding properties of antibodies.

Variability in the third hypervariable region of the dextran antibodies arises from germ-line diversity (J<sub>H</sub> segments), by combinatorial joining of V<sub>H</sub> and J<sub>H</sub> (and possibly D<sub>H</sub>) segments and by the mechanism for producing D segment diversity. Of these, D segment diversity appears to be by far the most extensive and, presumably, important in generating a diversity of antigen-binding sites (Figs 2, 3).

One striking feature evident in Fig. 3 is that immunoglobulins binding particular haptens share similar V<sub>H</sub> segments, exhibit D segments of similar size (for example, zero for levans, four for galactans, and so on), and to a lesser extent share similar J<sub>H</sub>

segments. Whether these correlations arise through some intrinsic properties of gene organisation, somatic mutational mechanisms or antigen selection is unknown.

### Correlation of dextran idiotypes and protein structure

Several interesting conclusions can be drawn about the correlation of V<sub>H</sub> structure and the various idiotypes of dextran-binding antibodies (Fig. 1). First, the individual idiotypes appear to correlate with the D segment sequence. The V<sub>H</sub> regions from the M104E and J558 proteins differ only by their D segments. As the light chains are identical, the individual idiotypes of M104E and J558 must be determined by positions 100 and 101. Moreover, the V<sub>H</sub> region of Hdex9, which differs from that of J558 by four residues and is identical at positions 100 and 101, has the individual J558 idiotypic. Likewise, the V<sub>H</sub> region of Hdex8, which differs from that of M104E by seven residues and is identical in the D segment, also has the individual M104E idiotypic. However, a single residue substitution can eliminate the individual idiotypic as demonstrated by the observation that Hdex3 is lacking both the M104E and J558 individual idiotypes (Figs 1, 2). Partial and in one case complete IdI reactivity can be retained by D segments sharing one of two residues (Hdex1, Hdex2 and Hdex7). The overall conclusion is that the individual idiotypes of myeloma proteins M104E and J558 are determined by residues 100 and 101 and, accordingly, are either encoded by a third gene segment (D) or represent antigenic determinants generated by a novel somatic mutational mechanism. Second, the IdX idiotypic correlates with several residues in the V<sub>H</sub> segment. The V<sub>H</sub> region from one IdX-negative protein, Hdex10, has been sequenced. Hdex10 differs from the two myeloma V<sub>H</sub> regions by four residues—positions 54, 55, 100 and 101. The substitution at position 55 destroys the attachment site for the carbohydrate moiety, distinguishing it from the other

| Protein            | Specificity       | V segment                             | D segment  | J segment                    | References |
|--------------------|-------------------|---------------------------------------|------------|------------------------------|------------|
| M104E              | 1,3 Dextran       | 75 80 90 95 SSSTAYMQLNSLTSEDSAVYYCARD | 100 101 YD | 102 110 117 WYFDVWGAGTTVTVSS | 32         |
| Hdex8              | "                 | ---G-----                             | YD         | -----                        | This paper |
| J558               | "                 | -----                                 | RY         | -----                        | "          |
| Hdex9              | "                 | --N--F-----                           | RY         | -----                        | "          |
| Hdex10             | "                 | -----                                 | VN         | -----                        | "          |
| Hdex6              | "                 | -----                                 | SH         | -----                        | "          |
| Hdex3              | "                 | -----                                 | R-         | -----                        | "          |
| Hdex7              | "                 | -----                                 | A-         | -----                        | "          |
| Hdex2              | "                 | -----                                 | NY         | -----                        | "          |
| Hdex1              | "                 | -----                                 | NY         | H---V-----                   | "          |
| Hdex5              | "                 | -----                                 | SN         | Y---Y-Q---L---               | "          |
| Hdex4              | "                 | -----                                 | K-         | Y---Y-Q---L---               | "          |
| T15,S63,S107,Y5236 | Phosphorylcholine | -Q-IL-L-M-A-RA--T-I-----              | YYGSSY     | -----                        | 46         |
| H8                 | "                 | -Q-IL-L-M-A-RA--T-I-----              | ---N--     | -----                        | "          |
| W3207              | "                 | -Q-IL-F-M-A-RA--T-I-----N             | --KYDL     | --V-----                     | "          |
| M603               | "                 | -Q-IL-L-M-A-RA--T-I-----N             | ---T       | -----                        | 47         |
| M167               | "                 | -Q-VL-L-M-A-RA--T-T---T---            | ADYGDSTYF  | G-----                       | 48         |
| M511               | "                 | -Q-IL-L-M-A-RA--T-I-----              | GDTG-SY    | -----                        | "          |
| A4                 | 2,1 Levan         | -K-SV-L-M-N-RA--TGI---TTG             | [ ]        | [ ]-AY--Q--L---              | 49         |
| E109               | "                 | -K-SVFL-M-N-RA--TGIH---TTG            | [ ]        | [ ]-AY--Q--L---              | "          |
| U61                | "                 | -K-SV-L-M-N-RA--TGI---TTG             | [ ]        | [ ]-AY--Q--L-P-              | "          |
| A47N               | "                 | -K-SV-L-M-N-RA--T-I---STG             | [ ]        | [ ]-AY--Q--L---              | "          |
| M315               | DNP               | -ENQFFLK-D-V-[ ]T-T---G-              | NDH        | L---Y--Q--L---               | 50         |
| M21                | Unknown           | PKN-LFL-MT--R---T-M---H               | GNYPW      | YAM-Y--Q--S-----             | 51         |
| M173               | Unknown           | AKN-L-L-MSKVR---T-L-----S             | PY         | YAM-Y--Q--S-----             | 52         |
| T601               | Galactan          | AKN-L-L-MSKVR---T-L-----G             | GYT        | G-----                       | 35         |
| X44                | "                 | AKN-L-L-MSKVR---T-L-----L             | H--        | G-AAAY--Q--L---A             | "          |
| X24                | "                 | AKN-L-L-MSKVR---T-L-----G             | ---        | G---Y--Q--L---               | "          |
| J539               | "                 | AKNSL-L-MSKVR---T-L-----L             | H--        | G-NAY--Q--L---A              | "          |

**Fig. 3** A comparison of published heavy chain sequences including the C-terminal portions of V<sub>H</sub> regions. The division of this region into V<sub>H</sub> segments, D segments, and J<sub>H</sub> segments is based on homology with the heavy chains binding dextran. The prototype sequence for the V<sub>H</sub> and J<sub>H</sub> segments is M104E. The prototypes for the D segments are the first sequence in each group. The J segments which do not extend to amino acid 117 are due to incomplete amino acid sequence data and not to shorter J segments.

11  $V_H$  regions (Fig. 1). It is attractive to postulate that the IdX idiotypic depends on amino acids at positions 54 and 55. This supposition is supported by the observation that Hdex8, which has greatly reduced IdX reactivity, has a substitution at position 54. Analysis of  $V_H$  regions from several additional IdX-negative antibody molecules are in progress to determine whether they also exhibit substitutions at positions 54 and 55 and to test the intriguing possibility that the IdX idiotypic may be in part associated with a carbohydrate moiety.

Thus, idiotypes can apparently arise from V segments (IdX) or D segments (IdI). These data on the hybridoma antibodies binding  $\alpha$ -1,3 dextran do allow us to assign structural correlates for the cross-reactive (V segment) and individual (D segment) idiotypes of several dextran antibodies. However, an idiotypic localised to a particular region (for example, the IdIJ558 to positions 100 and 101) may require particular sequences in still other portions of the V region for its expression (for example, V and J segments like those found in dextran-binding antibodies). Accordingly, the interpretation of genetic mapping of idiotypes is complex. Indeed, one can never be certain what is being mapped—a V segment, a D segment, a J segment, the ability to produce certain somatic variants, or some combination of these.

In an historical context, note that Kabat and coworkers predicted the existence of minigenes for each hypervariable region based on an analysis of the V region amino acid sequences<sup>13,36</sup>. In addition, Capra and Kindt postulated the existence of multiple V coding elements based on an analysis of  $V_H$  sequence data and idiotypes<sup>37,38</sup>. If the D segments are encoded by distinct gene segments, they are 'minigenes' that encode the individual idiotypes of murine antibodies binding dextran.

## The evolution of split-gene segments and DNA rearrangements

The  $V_H$  regions and  $V_L$  regions are homologous to one another at the protein level<sup>31,39</sup>. Both  $V_L$  and  $V_H$  are encoded by V and J gene segments of similar size. This homology implies that the split-gene strategy and the mechanism for rearranging split genes must have arisen before the divergence of light and heavy chains. Indeed, it is attractive to speculate that split genes and mechanisms for their rearrangement evolved before the emergence of the vertebrate immune system, possibly in families of genes coding membrane recognition functions. It will be interesting to determine whether there are other contemporary

multigene families using strategies of split genes and DNA rearrangement for the amplification of information<sup>5</sup>. This is discussed elsewhere<sup>40</sup>.

The patterns of phenotypic diversity in the  $V_H$  regions from antibodies binding  $\alpha$ -1,3 dextran have allowed us to make several important conclusions. We can define  $V_H$  and  $J_H$  segments which are homologous to their light chain counterparts. Diversity in a third region, the D segment, can be explained by a novel somatic mutational mechanism, the existence of a third germ-line (D) gene segment, or the less likely possibility of a large number of  $J_H$  gene segments. Antibody diversity appears to arise from several sources: multiple germ-line gene segments, combinational joining of  $V_H$  and  $J_H$  gene segments and the

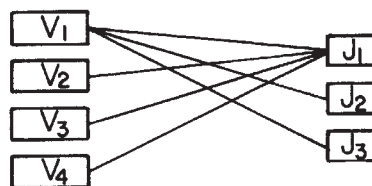


Fig. 4 A diagram indicating the combinational joining of  $V_H$  and  $J_H$  segments.  $V_1$  to  $V_4$  and  $J_1$  to  $J_3$  are defined in Fig. 2.

mechanism for producing D segment diversity which includes extensive nucleotide substitutions as well as the insertion or deletion of multiple residues. Correlations between the  $V_H$  sequences and the idiotypes of the antibodies to  $\alpha$ -1,3 dextran are possible but there should be caution in the interpretation of genetic mapping studies which use idiotypes. Studies are in progress in our laboratories to expand our analyses of the phenotypic (protein) diversity in the dextran system and to analyse the gene segments encoding the dextran response.

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# A UGA termination suppression tRNA<sup>Trp</sup> active in rabbit reticulocytes

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*A tRNA<sup>Trp</sup> was purified from rabbit reticulocytes which suppresses the UGA termination codon of  $\beta$ -haemoglobin mRNA. Evidence is presented that the  $\beta$ -haemoglobin readthrough protein is found in reticulocyte translations and intact cells. Some natural readthrough proteins perform essential functions; they are synthesised through suppression of UGA or UAG but not UAA termination codons.*

THE termination of polypeptide chain synthesis is signalled by the three codons UAG (amber), UAA (ochre) and UGA (opal). Termination is carried out through the activity of release factors which are thought to recognise termination codons and release the nascent polypeptide chain<sup>1-3</sup>. In some cases, termination is suppressed by a tRNA which recognises one of the termination codons and inserts an amino acid<sup>4</sup>. The ribosome then continues adding amino acids to the nascent chain until it encounters the next termination codon. Proteins which are synthesised in this manner are called 'readthrough' proteins and the tRNAs which recognise the termination codons are called suppressor tRNAs. Suppressor activity is of two types; naturally occurring and induced through mutagenesis. Natural suppression seems to be a mechanism for producing proteins which, while related to other proteins, nonetheless perform distinct and essential functions. Naturally occurring suppression has been largely associated with viral infection in prokaryotic as well as eukaryotic systems. The example of natural readthrough which has been studied in greatest detail is Q $\beta$  infection of *Escherichia coli*. The coat protein gene has a UGA termination codon. Wild-type *E. coli* tRNA<sup>Trp</sup> misreads the UGA codon at a low frequency, producing a coat readthrough protein<sup>5-8</sup> which is required for infection by Q $\beta$ <sup>9</sup>. An analogous situation may exist in bacteriophage  $\lambda$  infection of *E. coli* where the UGA termination codon of the O protein is read through<sup>10,11</sup>. Filamentous bacteriophages may also require UGA readthrough<sup>11</sup>. Recently, a readthrough protein produced by suppression of a UAG termination codon has been detected during the infection of tobacco plants by tobacco mosaic virus (TMV)<sup>12</sup>. However, this suppressor activity has not yet been associated with a particular tRNA.

Here we report a UGA suppressor activity in rabbit reticulocytes which produces a  $\beta$ -haemoglobin readthrough protein. A purified rabbit reticulocyte tRNA<sup>Trp</sup> dramatically enhances UGA suppression in the reticulocyte lysate. Furthermore, evidence is presented which strongly suggests that the UGA suppressor activity is active within intact reticulocyte cells producing very small amounts of the  $\beta$ -haemoglobin readthrough protein. This is the first demonstration of naturally occurring readthrough in a non-viral system.

## Detection of suppression

Suppressor tRNAs from prokaryotic systems have been well characterised and sequenced. Although yeast suppressor tRNAs function in higher eukaryotic lysates<sup>13,14</sup> prokaryotic

suppressor tRNAs often do not work in eukaryotic systems<sup>13</sup>. Our assumption was that this was due to the inability of eukaryotic aminoacyl tRNA synthetases to aminoacylate these tRNAs efficiently. Accordingly, we devised an *in vitro* system in which a purified prokaryotic tRNA and its purified cognate prokaryotic synthetase are added to a eukaryotic cell-free protein synthesising system. The well characterised rabbit reticulocyte system was used in which haemoglobin is the predominant (90-95%) product synthesised. Figure 1 shows reticulocyte translations using <sup>35</sup>S-methionine analysed on an SDS gel. Lane *a* shows the effect of adding the *E. coli* UGA suppressor without synthetase while lane *b* shows the effect of adding the UGA suppressor tRNA and *E. coli* Trp synthetase. A new band (*B*) appears which is somewhat larger than the two globin chains (Hb). Lane *c* shows the effect of adding an *E. coli* UAA suppressor tRNA and *E. coli* Tyr synthetase. A different protein (*A*) is produced which migrates more slowly than haemoglobin. Lane *d* shows the effect of adding the UAA suppressor tRNA without synthetase. Lane *e* is a control with no additions. The readthrough proteins *A* and *B* have distinct mobilities even when mixed together (data not shown).

$\alpha$ -Haemoglobin mRNA terminates in a UAA codon and the next termination codon in phase occurs 21 codons downstream<sup>15</sup>. This suggests that band *A* seen in Fig. 1 is the  $\alpha$ -haemoglobin readthrough protein. Rabbit  $\beta$ -haemoglobin mRNA terminates in a UGA termination codon. The next termination signals, two UAAs in tandem, occur 22 codons later<sup>16</sup>. This suggests that band *B* in Fig. 1 is the  $\beta$ -haemoglobin readthrough protein.  $\alpha$ - And  $\beta$ -haemoglobin readthrough proteins have also been synthesised when the appropriate yeast suppressor tRNAs are added to a wheat-germ extract<sup>17</sup>. In further experiments not shown here, an *E. coli* UAG suppressor tRNA and its purified tyrosine synthetase were added to the reticulocyte lysate but no readthrough bands were detected. The UAG suppressor was shown to function in the reticulocyte lysate using an RNA from a Q $\beta$  mutant (*am* - 1) which has a UAG nonsense mutation in the synthetase gene<sup>14</sup>.

We observed faint bands in all of the lanes of Fig. 1 at the same position as the putative  $\beta$ -haemoglobin readthrough protein. This suggested that there might be natural readthrough of the UGA termination codon of rabbit  $\beta$ -haemoglobin mRNA. Before proceeding further, we carried out additional analysis of the readthrough band to demonstrate that it is in fact the  $\beta$ -haemoglobin readthrough protein.  $\alpha$ - And  $\beta$ -haemoglobin chains and the  $\beta$ -haemoglobin readthrough protein were purified then subjected to tryptic digestion<sup>18</sup>. The results are shown in Fig. 2 and a diagram shows the position of the methionine-containing tryptic peptides in both  $\alpha$ - and  $\beta$ -haemoglobin and their readthrough proteins<sup>15,16</sup>.  $\alpha$ -Haemoglobin has one methionine-containing tryptic peptide composed of 9 amino acids. There are no additional methionines in its readthrough protein.  $\beta$ -haemoglobin has one methionine-containing tryptic peptide composed of 19 amino acids. That peptide, which ends in lysine, is followed by the sequence

Val-Lys at its C terminus; in the case of incomplete cleavage at the first lysine residue, one may get a peptide containing 21 amino acids. The readthrough protein of  $\beta$ -haemoglobin has an additional methionine-containing tryptic peptide, 7 amino acids in length<sup>16</sup>.

The tryptic peptides were analysed by electrophoresis at pH 3.5. Lane *a* of Fig. 2 has <sup>35</sup>S-methionine alone, lane *b* contains the tryptic digest of  $\beta$ -haemoglobin. The direction of migration is to the left (towards negative) and two spots are seen. This suggests that the slower spot contains 19 amino acids while the faster spot contains 21 amino acids, resulting from incomplete cleavage at the C-terminal lysine. Incomplete cleavage results in the addition of Val-Lys to the peptide and a doubling of its positive charge at pH 3.5. This assignment is strengthened by the observation that longer digestion periods yield more of the slower spot. The tryptic digest of  $\beta$ -haemo-

globin was also analysed by chromatography<sup>18</sup>. The results of this experiment (not shown) yielded two spots which migrated at positions very close to each other, suggesting the general properties of these two peptides, including both length and chemical characteristics, are very similar. This is consistent with our identification of the second minor peptide as arising from incomplete digestion by trypsin. Lane *c* shows the tryptic digest of  $\alpha$ -haemoglobin which yields one major spot. Lane *f*, a mixture of tryptic digests of  $\alpha$ - and  $\beta$ -haemoglobin show three spots. The tryptic peptides of the putative  $\beta$ -haemoglobin readthrough protein are shown in lane *d*. This produced three spots, two of which migrated at the same position as the  $\beta$ -haemoglobin spots and one additional spot migrating between the two. Lane *e* shows a mixture of the tryptic digests of both  $\alpha$ - and  $\beta$ -haemoglobin and the  $\beta$ -haemoglobin readthrough protein. Four spots are now observed. The conclusion from this experiment is that band B in Fig. 1 is indeed the  $\beta$ -haemoglobin readthrough protein as shown by the appearance of an additional methionine containing tryptic peptide which is expected in view of the mRNA sequence after the UGA termination codon.

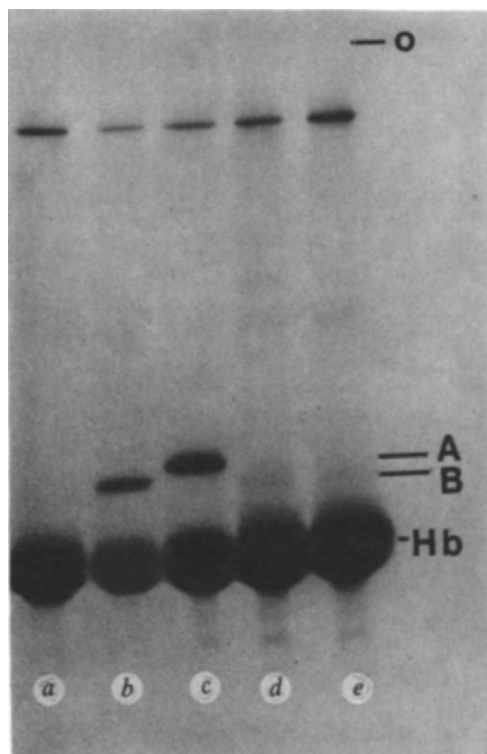
### UGA suppression in reticulocyte lysate

Experiments were carried out to detect natural readthrough producing the  $\beta$ -haemoglobin readthrough protein. Two-dimensional gel electrophoresis<sup>19</sup> was used to separate the various products made by the reticulocyte lysate. Figure 3*a* shows a 12-hour exposure of a two-dimensional gel of a translation with *E. coli* UGA suppressor tRNA (hereafter all translation with *E. coli* suppressor tRNA also contained the appropriate *E. coli* synthetase). The small spot indicated by the vertical arrow is the  $\beta$ -haemoglobin readthrough protein while the larger spots below it are haemoglobin chains. Figure 3*b* shows a 1-week exposure of the same gel. Figure 3*c* is a 1-week exposure of a two-dimensional gel of a translation with no additions. A faint spot appears at the same position as the  $\beta$ -haemoglobin readthrough protein. This gel also revealed a few proteins with the same molecular weight as the  $\beta$ -haemoglobin readthrough protein but very different isoelectric points. These proteins prevented the purification of enough natural  $\beta$ -haemoglobin readthrough protein to permit tryptic analysis.

To ensure identification of this weak spot another electrophoretic experiment was carried out; a two-dimensional gel was run using isoelectric focusing in the first dimension and an acid-urea gel in the second<sup>20</sup>. The  $\beta$ -haemoglobin readthrough protein was detected by autoradiography. The gel fragment containing this spot was excised, rehydrated, and loaded on to an SDS gel for further analysis. A gel slice containing haemoglobin was excised from an SDS gel to run in the system as a marker. The control in Fig. 3*D*, lane *a*, contains two bands, one of which is haemoglobin. Since all the protein in lane *a* is haemoglobin, the second band is likely to be a dimer of two haemoglobin chains. Crosslinking could have occurred during either staining, destaining, drying or autoradiography. Lane *b* came from a translation containing the *E. coli* UGA suppressor analysed as described above; in addition to haemoglobin and haemoglobin dimers, it contains the  $\beta$ -haemoglobin readthrough protein labelled B. Lane *c* was a translation with no additions analysed on a two-dimensional gel. This lane also shows a weak band which co-migrates with the  $\beta$ -haemoglobin readthrough protein. This evidence strongly suggests that there is a low level of natural readthrough of the UGA termination codon of  $\beta$ -haemoglobin mRNA in the rabbit reticulocyte lysate.

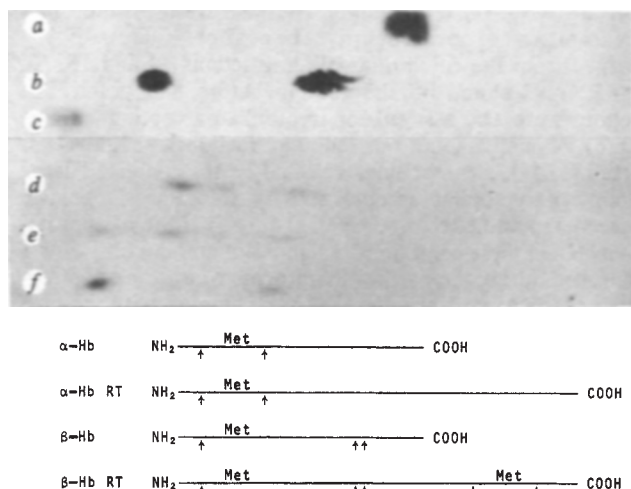
### Readthrough proteins in rabbit reticulocyte cells

An experiment was carried out to detect synthesis of the  $\beta$ -haemoglobin readthrough protein in intact rabbit reticulocyte cells. Reticulocytes were incubated in a cell culture medium in the presence of radioactive amino acids<sup>21</sup>. The proteins were separated on two-dimensional gels<sup>19</sup>. Figure 4*a* shows an analysis of the mixture of the incubation and a translation with



**Fig. 1** Autoradiogram of rabbit reticulocyte lysates incubated with <sup>35</sup>S-methionine and analysed on an SDS gel. *E. coli* suppressor tRNAs were isolated from *E. coli* DB 6437 Sup C (UAA)<sup>33</sup> and LS 849 Su<sup>7+</sup> (UGA)<sup>34</sup> (provided by David Botstein). LS 849 was grown as described in ref. 35. An S-30 supernatant was prepared<sup>36</sup> and then centrifuged at 100,000g for 5 h. The supernatant was extracted with phenol saturated with 10 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 30 mM NH<sub>4</sub>Cl. After deacylation<sup>37</sup> and exhaustive dialysis against 200 mM NaAc (pH 5.0), the tRNA<sup>Tyr</sup> (ref. 38) and tRNA<sup>Trp</sup> (refs 23, 24) were purified and stored at -60 °C. *E. coli* Tyr aminoacyl tRNA synthetase<sup>39</sup> (Tyr R.S.) and *E. coli* Trp aminoacyl tRNA synthetase<sup>23</sup> (Trp R.S.) were gifts from P. Schimmel and K. Muench respectively. One part haemin and 0.4 parts creatine phosphokinase<sup>40</sup> were added to 100 parts reticulocyte lysate<sup>41</sup>. Reticulocyte translation solution (25  $\mu$ l) contained 0.5 mM spermidine, 8 mM creatine phosphate, 25  $\mu$ M amino acids except methionine, 2 mM dithiothreitol (DTT), 20 mM HEPES (pH 7.6), 0.65 mM MgAc<sub>2</sub>, 80 mM KAc, 5  $\mu$ l (5 mCi ml<sup>-1</sup>) <sup>35</sup>S-methionine (Amersham), and 10  $\mu$ l reticulocyte lysate. 5  $\mu$ g tRNA and 0.2  $\mu$ g aminoacyl tRNA synthetase were added as indicated below. Reaction mixtures were incubated at 37 °C for 1 h and stored at -20 °C for subsequent use. Reaction mixtures (1  $\mu$ l) contained approximately 100,000 trichloroacetic acid (TCA) precipitable counts. Aliquots of 2  $\mu$ l were loaded into each lane of a 15–20% linear gradient polyacrylamide SDS gel<sup>42</sup>. The gel was run for 1 h after the bromophenol blue (BPB) tracking dye ran off the bottom. The gel was stained, destained, fluorographed<sup>43</sup> and then exposed to XR-5 film for 1 d. Lane *a*, Su<sup>7+</sup> (UGA) tRNA; lane *b*, Su<sup>7+</sup> (UGA) tRNA and *E. coli* Trp R.S.; lane *c*, Sup C (UAA) and *E. coli* Tyr R.S.; lane *d*, Sup C (UAA) tRNA; lane *e*, no additions. The origin is marked O.



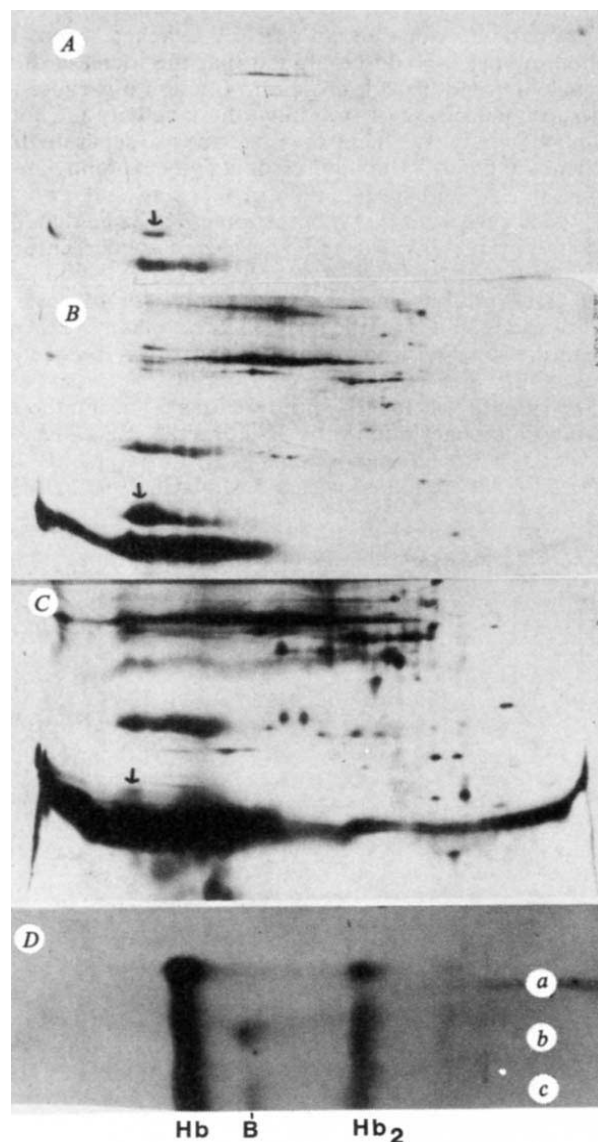


**Fig. 2** Autoradiogram of tryptic digestion of various haemoglobin chains. At the bottom is a diagram showing the  $\alpha$ - and  $\beta$ -haemoglobin (Hb) chains as well as their readthrough (RT) proteins. The vertical arrows indicate the positions of tryptic digestion sites around methionine-containing peptides. A 25- $\mu$ l translation mixture was run on a 15% polyacrylamide SDS phosphate gel<sup>44</sup> for 20 h at 80 mA to separate <sup>35</sup>S-methionine-labelled  $\alpha$ - and  $\beta$ -haemoglobin. A 25- $\mu$ l translation mixture with Su<sup>7+</sup> (UGA) tRNA and *E. coli* Trp R.S. was run on an SDS gel<sup>42</sup> as in Fig. 1 to purify the putative  $\beta$ -haemoglobin readthrough protein. Gels were autoradiographed to locate the protein. Samples were prepared and digested with trypsin as described<sup>18</sup> with the following modifications; 4  $\mu$ l of 25 mg ml<sup>-1</sup> bovine serum albumin was added to the  $\beta$ -haemoglobin readthrough protein sample; the  $\alpha$ - and  $\beta$ -haemoglobin samples required no carrier. After the final lyophilisation, the samples were taken up in 20  $\mu$ l H<sub>2</sub>O. The tryptic peptides were analysed by electrophoresis on cellulose-coated thin layer plates for 90 min at 1,000 V, 25 mA in pyridine:acetic acid:H<sub>2</sub>O, 1:10:100 (pH 3.5). The plate was immersed in PPO- $\beta$ -naphthalene<sup>45</sup> and then exposed to film for 2 d except for lane c which was exposed for 1 d. Lane a, 3,000 c.p.m. <sup>35</sup>S-methionine. The following tryptic digests: lane b, 3,000 c.p.m.  $\beta$ -haemoglobin; lane c, 3,000 c.p.m.  $\alpha$ -haemoglobin; lane d, 1,500 c.p.m.  $\beta$ -haemoglobin readthrough protein; lane e, 500 c.p.m.  $\alpha$ -haemoglobin and 500 c.p.m.  $\beta$ -haemoglobin and 500 c.p.m.  $\beta$ -haemoglobin readthrough protein; lane f, 750 c.p.m.  $\alpha$ -haemoglobin and 750 c.p.m.  $\beta$ -haemoglobin. The origin is marked O.

an *E. coli* UGA suppressor; the 1-week exposure shows a well developed spot in the position of the  $\beta$ -haemoglobin readthrough protein as indicated by the arrow. Figure 4b is a 1-month exposure of just the incubation of intact cells; several additional spots appeared after the longer exposure. There is a small spot which migrates at the position of the  $\beta$ -haemoglobin readthrough protein. Because of the low level of the putative  $\beta$  readthrough protein in intact cells, no further analysis was attempted. It seems likely that there is a low level of production of the  $\beta$ -haemoglobin readthrough protein in intact reticulocyte cells.

### tRNA fractionation and suppressor activity

The results presented above suggested that there might be a tRNA in the reticulocyte cell which suppresses the UGA termination codon. Reticulocyte tRNA was fractionated on BD cellulose<sup>22</sup>, widely used in tRNA purification, to purify this activity. As shown in the graph at the top of Fig. 5, the bulk of the tRNA emerges in the first third of the column. The assay shown for tryptophan acceptor activity has a maximum near fraction 13, and a leading shoulder in fractions 5–8. Each of these fractions was precipitated and then redissolved in H<sub>2</sub>O. A sample of 4  $\mu$ g of tRNA from each fraction was added to a 25  $\mu$ l reticulocyte translation representing a 10–20% increase in the amount of tRNA in the reaction. The results are shown at the bottom of Fig. 5. The lanes marked Su<sup>+</sup> and Su<sup>-</sup> were controls with the *E. coli* UGA suppressor added and with no additions, respectively. The Su<sup>+</sup> lane shows the  $\beta$ -haemoglobin readthrough protein which is enhanced in fractions 5–8. In fraction 6 a number of other bands are also enhanced for an unknown



**Fig. 3** Autoradiograms of <sup>35</sup>S-methionine-labelled reticulocyte translation mixtures analysed in various gel systems. Two-dimensional gels<sup>19</sup> were run with pH 6–8 and pH 3–10 ampholytes in the ratio of 4:1 in isoelectric focusing. The second dimension (vertical) was a 15–20% linear gradient polyacrylamide SDS gel. The gel was treated as in Fig. 1. a, 5  $\times$  10<sup>5</sup> c.p.m. of a translation mixture with Su<sup>7+</sup> (UGA) tRNA and *E. coli* Trp R.S. The film was exposed for 12 h. b, 1-week exposure of gel a. c, 1.5  $\times$  10<sup>6</sup> c.p.m. of a translation mixture with no additions. The film was exposed for 1 week. d, Readthrough spots were isolated from isoelectric focusing acid-urea two-dimensional gels and run on SDS gels. Isoelectric focusing tube gels were run as above<sup>19</sup>. The gels were equilibrated in buffer O' (5% HAc, 10% glycerol) for 30 min and then stored at -60 °C or used immediately. Slab gels of 15% polyacrylamide acid-urea<sup>20</sup> were pre-run for 1 h at 125 V. The equilibrated tube gels were loaded on top of acid-urea gels and overlaid with freshly made 1% agarose in buffer O' in the same manner as the second dimension of two-dimensional O'Farrell gels<sup>19</sup>. Gels were run for 20 h at 125 V, destained, dried and autoradiographed for 1 week. The appropriate gel cubes were located, excised, rehydrated in buffer O' for 30 min, and then analysed on an SDS gel<sup>42</sup> as in Fig. 1. Only the SDS gel is shown: lane a, the haemoglobin band from a translation mixture was isolated from an SDS gel run as in Fig. 1. The gel slice was rehydrated in buffer O and loaded on to the SDS gel. The control haemoglobin now appears as two spots, probably due to dimerisation. Lane b, the  $\beta$ -haemoglobin readthrough spot from 5  $\mu$ l of a translation mixture with Su<sup>7+</sup> (UGA) tRNA and *E. coli* Trp R.S. was isolated from an isoelectric focusing acid-urea two-dimensional gel. Lane c, 25  $\mu$ l of a translation mixture with no additions was assayed in a way analogous to b. The gel was exposed to film for 2 months.

reason. These bands are all detected in reticulocyte translations with no additions on longer exposures; the bands are resistant to RNase digestion (results not shown) so they are not polypeptide chains prematurely released with tRNA attached. From this experiment alone, it could be argued that the increase of the  $\beta$ -haemoglobin readthrough protein in fraction 6 is caused by the general enhancement of protein synthesis activity and not by a suppressor activity. We therefore undertook to separate these two activities and purify the suppressor activity to homogeneity.

The fact that the enhancement occurred on a shoulder of the tRNA<sup>Trp</sup> peak suggested that suppression might be mediated by a tRNA<sup>Trp</sup>. This was investigated by a second column chromatography of aminoacylated tryptophanyl-tRNA<sup>Trp</sup> on BD-cellulose. BD-cellulose has a strong affinity for planar ring molecules and binds reticulocyte tryptophanyl-tRNA<sup>Trp</sup> because of the attached aromatic amino acid; this is the standard method for purifying *E. coli* tRNA<sup>Trp</sup> (refs 23, 24). As shown in Fig. 6, the column was loaded with fractions 5–8 from the first column after aminoacylation with <sup>3</sup>H-Trp. Fractions 3–13 were eluted with 0.8 M NaCl to wash off all uncharged tRNAs. Fractions 14–18 were eluted with 1.5 M NaCl, 20% EtOH to

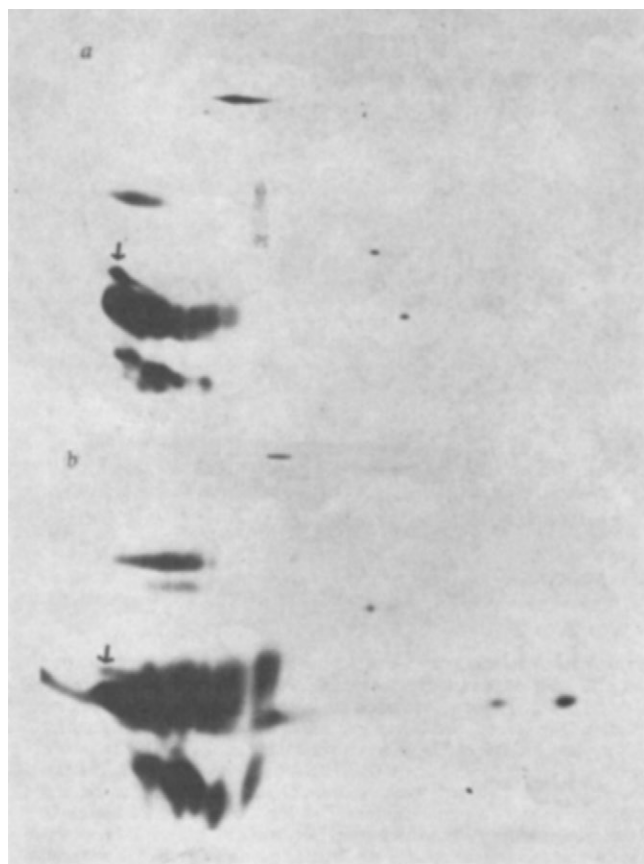
wash all tRNA off the column. The final wash resulted in a significant peak of optical density. The peak of <sup>3</sup>H-Trp-tRNA<sup>Trp</sup> emerged from the column largely in fractions 14 and 15. No Trp-tRNA<sup>Trp</sup> eluted off the column before that point. The fractions were also assayed for tryptophan-accepting activity, (Fig. 6). A small amount was found in fractions 1–13, however fractions 14–17, comprising the final wash, contained large amounts of tryptophan-acceptor activity. Since the ester linkage between Trp and tRNA<sup>Trp</sup> is labile with a half life of approximately 6 h in these conditions, a significant amount of tRNA<sup>Trp</sup> is released during the purification process which took approximately 10 h. Almost half the tRNA<sup>Trp</sup> came off with the final wash.

The autoradiogram shown in Fig. 6 shows the effect of these various fractions on translation in the reticulocyte system. There is a small enhancement of readthrough in many of fractions 1 to 15. However, a very large increase in readthrough is observed in fractions 16 and 17. Interesting, in fractions 14–17, there is high tryptophan-acceptor activity. Figure 5 shows the reticulocyte suppressor tRNA<sup>Trp</sup> and the nonsuppressing tRNA<sup>Trp</sup> were incompletely separated; it is likely that the second column achieved a better separation. The total radioactivity incorporated in translations of fractions 16 and 17 in Fig. 6 was somewhat less than that found in translations containing other fractions. This is probably associated with a higher amount of salt in that fraction which was not fully washed out. Longer exposure of the autoradiogram in Fig. 6 revealed that the general stimulatory activity migrated with the major peak of tRNA, fractions 2–5. A densitometer tracing of the optical density in the  $\beta$ -haemoglobin readthrough protein band compared to the haemoglobin band indicated that the intensity of the bands is approximately equal. The haemoglobin band contains a mixture of both  $\beta$  and  $\alpha$  chains, while the readthrough band contains only  $\beta$ -haemoglobin readthrough protein. This suggests that a very high efficiency of readthrough is produced by fractions 16 and 17. It appears more efficient than the *E. coli* UGA suppressor (lane Su<sup>+</sup>). Electrophoresis on two-dimensional gels showed that the readthrough protein synthesised in translations containing fractions 16 and 17 comigrated with the  $\beta$ -haemoglobin readthrough protein and migrated differently from the  $\alpha$ -haemoglobin readthrough protein in both dimensions (results not shown).

## Discussion

Analysis of translations with no additions revealed a small amount of a peptide which had the identical mobility as the  $\beta$ -haemoglobin readthrough protein in three different electrophoretic systems. This strongly suggested the presence of a UGA suppressor tRNA in the reticulocyte. This was examined by fractionating reticulocyte tRNA and isolating a peak which enhanced the production of the  $\beta$ -haemoglobin readthrough protein. This was identified as a tRNA<sup>Trp</sup> by separating tryptophanyl-tRNA<sup>Trp</sup> from all other tRNAs. Proteins from intact rabbit reticulocyte cells incubated with <sup>35</sup>S-methionine were analysed by two-dimensional gel electrophoresis. A small spot was detected in the same position as authentic  $\beta$ -haemoglobin readthrough protein. Although this identification does not establish proof, coupled with the isolation of a tRNA from the reticulocyte cell which reads the UGA termination codon, this result strongly suggests that we have detected a low level of  $\beta$ -haemoglobin readthrough protein in the intact reticulocyte cell.

The  $\beta$ -haemoglobin readthrough protein appears to be stable in *in vitro* incubations. Additional incubation in the presence of glucose and/or ATP did not degrade the  $\beta$ -haemoglobin readthrough protein nor did its position change in two-dimensional gel electrophoresis which, for example, might occur as a result of glycosylation. The readthrough activity in the TMV<sup>12</sup> and Q $\beta$ <sup>25</sup> systems are sensitive to changes in Mg<sup>2+</sup> concentration. The concentrations of Mg<sup>2+</sup>, K<sup>+</sup> or Na<sup>+</sup> have little influence on the amount of  $\beta$ -haemoglobin readthrough protein produced (data not shown). The material which produces the



**Fig. 4** Autoradiographs of two-dimensional gels analysing labelled reticulocyte proteins. Reticulocytes were incubated<sup>21</sup> with the following modifications: the 10-ml incubation mixture contained Krebs-Ringer phosphate, 0.001% heparin, 2 ml dialysed calf serum, 0.2 mM cysteine, 2 mM glutamine, 0.05 mM tryptophan, 0.2 mM asparagine, 10 mM 2-mercaptoethanol (2-ME), 1 mg ml<sup>-1</sup> glucose, 1.8 mCi <sup>35</sup>S-methionine and 250  $\mu$ Ci of a <sup>3</sup>H-labelled amino acid mixture (Amersham). After the incubation, the cells were centrifuged at 15,000g for 5 min, washed once in Krebs-Ringer phosphate, centrifuged, lysed in an equal volume of H<sub>2</sub>O containing 10 mM phenylmethylsulphonyl fluoride and then centrifuged for 20 min to remove cell membranes. The supernatant was precipitated with 10% TCA for 30 min at 0°C and centrifuged for 10 min. The pellet was redissolved in one volume of 10% glycerol, 10% SDS, 2 mM EDTA, 1 M DTT, 100 mM Tris-HCl (pH 7.5), 0.1% BPB and titrated to pH 7.0 with 3 M NaOH. The sample was boiled for 5 min and stored at -20°C for subsequent use. Two-dimensional gels<sup>18</sup> were run as in Fig. 3. *a*, 7.2  $\times$  10<sup>4</sup> c.p.m. of the intact cell incubation plus 2  $\times$  10<sup>5</sup> c.p.m. of an *in vitro* translation mixture with Su7- (UGA) tRNA and *E. coli* Trp R.S. The film was exposed to the gel for 1 week. *b*, 2  $\times$  10<sup>5</sup> c.p.m. of the intact cell incubation. The film was exposed to the gel for 1 month.



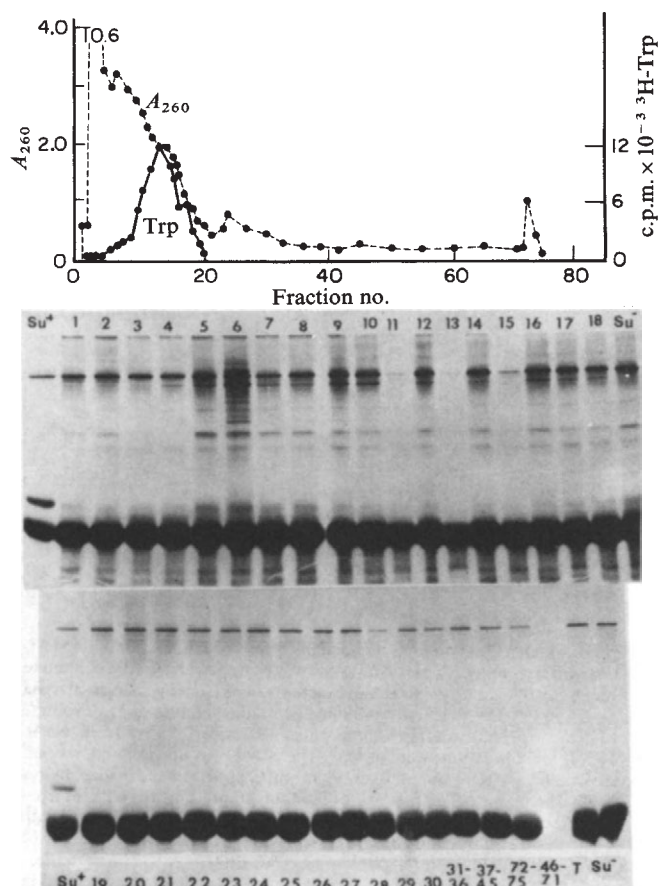
readthrough is likely to be RNA as KOH treatment abolishes production of the  $\beta$ -haemoglobin readthrough protein. The position of this RNA molecule on a column changes after it has been aminoacylated with tryptophan. It is on this basis that we feel confident that there is a reticulocyte tRNA<sup>Trp</sup> which reads the UGA termination codon. It has also been shown that tRNA<sup>Trp</sup> reads UGA in other systems<sup>5-8,26</sup>.

The tRNA species which has been purified is highly active in producing the  $\beta$ -haemoglobin readthrough protein; it is not likely that it is operating by making occasional mistakes. This tRNA is now being purified further with a view to determining its complete nucleotide sequence. This will allow us to determine whether it is reading UGA because of a mutation in the anticodon which makes it complementary to UGA or because of a difference in nucleotide modification from the more abundant reticulocyte nonsuppressing tRNA<sup>Trp</sup>. Methylation and other nucleotide modifications have been shown to alter the reading abilities of other tRNAs<sup>4</sup>. Misreading in this case requires an A·C base pair, therefore it cannot be described as wobble misreading.

Broadly considered, there are two different types of termination suppression. One is induced suppression, produced by mutagenesis, which has been studied extensively in prokaryotic and lower eukaryotic systems. A nonsense mutation produces a termination codon in the middle of the structural protein and a second mutation produces a termination suppressor tRNA which reads the termination codon restoring the original polypeptide. The second class are natural suppressors; these occur in the wild-type organism. These have not been studied extensively and our knowledge is relatively meagre. The best characterised example of naturally occurring suppression is the coat readthrough protein which is synthesised during infection of *E. coli* cells by Q $\beta$  virus; wild-type *E. coli* tRNA<sup>Trp</sup> suppresses the UGA termination codon of the coat protein<sup>5-8</sup>. The readthrough protein, produced at low frequency, is essential for the production of infective Q $\beta$  virions<sup>9</sup>. The function of the readthrough protein is not known. Another example of naturally occurring UGA suppression is found in bacteriophage  $\lambda$  infection of *E. coli* where the O protein is read through producing the readthrough protein O' (refs 10, 11). As in Q $\beta$  infection, the O' protein appears to be essential for infection<sup>11</sup>. Very little is known about natural readthrough in eukaryotic systems. A UAG (amber) suppressor tRNA may be used in the translation of a tobacco mosaic virus mRNA<sup>12</sup>. Addition of yeast amber suppressor tRNA to the reticulocyte system enhances the production of a 160,000 MW protein and diminishes a 110,000 MW protein. These proteins both have the same N-terminal sequence and have been isolated from the infected plant leaves. The 160,000 MW protein is produced in the rabbit reticulocyte lysate suggesting that it has a natural UAG suppressor activity. A UAG suppressor activity has also been inferred in the production of the polymerase protein in murine leukaemia virus infection<sup>27,28</sup> but appears not to be used in the related Rous sarcoma virus infection<sup>29</sup>.

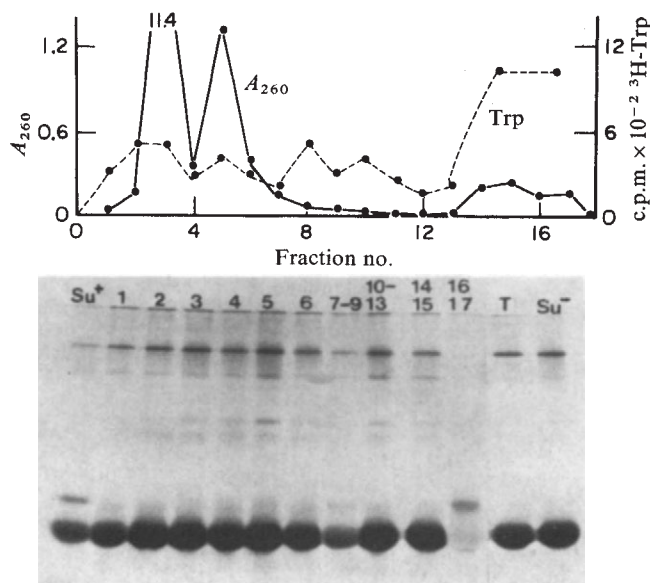
The UGA suppressor activity we have demonstrated in the reticulocyte is the first example of a natural suppressor activity not associated with viral infection. It suggests that naturally occurring termination suppressors may be active in non-viral systems. Further analysis will be necessary to determine the extent to which these naturally occurring suppressors are found in other systems. Preliminary results from experiments involving the addition of fractionated yeast tRNA to the reticulocyte lysate and translations of rabbit reticulocyte mRNA in the wheat-germ system indicate a UGA suppressor activity in both these sources.

The existence of haemoglobin molecules with C-terminal additions is not unknown. In humans, mutations are found in the termination codons of both  $\alpha$ - and  $\beta$ -haemoglobin mRNA (haemoglobins Constant Springs and Tak<sup>30</sup>). Apparently, the addition of a peptide at the C terminus does not grossly interfere with physiological function although not much is known about other effects it may have.



**Fig. 5** Fractionation of reticulocyte tRNA on BD-cellulose and an assay for the production of readthrough proteins. Reticulocyte lysate<sup>31</sup> was centrifuged at 100,000g for 5 h. The supernatant was extracted with phenol saturated with 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, then deacylated<sup>37</sup> and dialysed overnight against 200 mM NaAc (pH 5.0). The RNA was loaded on to a 12-ml BD-cellulose column<sup>22</sup> in 200 mM NaCl and developed with a linear 500 ml gradient from 0.4 to 0.9 M NaCl (fractions 1 to 71). This was followed by a 10% EtOH, 1 M NaCl wash (fractions 72 to 75). All column buffers contained 25 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>. Yeast Trp R.S. was isolated: 1 lb of baker's yeast was lysed with toluene<sup>46</sup> and then centrifuged at 13,000g for 40 min. The supernatant was made 100 mM Tris-HCl (pH 7.5), 30 mM 2-ME, 10 mM MgCl<sub>2</sub> and the pH was adjusted to 7.5 with 2 M NaOH. 0.1 volume of 1 M BaCl was added and the solution was centrifuged for 30 min. The supernatant was made 20 mg ml<sup>-1</sup> in streptomycin sulphate and centrifuged for 30 min. The supernatant was precipitated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (ref. 46) and the pellet redissolved with and dialysed against 20 mM KHPO<sub>4</sub> (pH 7.0), 1 mM DTT and stored in 50% glycerol at -20 °C. Charging assays were performed as described in ref. 46 on 8  $\mu$ l of each fraction for Leu, Tyr, Lys, Gly and Trp. Only the Trp acceptance curve is shown. Fractions were precipitated, washed twice with 66% EtOH, taken to near dryness under a vacuum, and redissolved in H<sub>2</sub>O to a concentration of 2  $\mu$ g  $\mu$ l<sup>-1</sup>. 2  $\mu$ l were added to a 25- $\mu$ l reticulocyte translation mixture which was incubated and analysed as in Fig. 1. The lane numbers refer to translations of the various fractions. The Su<sup>+</sup> lane contained Su<sup>+</sup> (UGA) tRNA and *E. coli* Trp R.S., the Su<sup>-</sup> lane had no additions. Lane 46-71 was inhibited by excess salt. Lane T contained a reaction with unfractionated reticulocyte tRNA. The top gel was exposed to film for 4 d and the bottom gel for 3 d.

A review of the literature on termination codons and natural suppressor tRNAs suggests that there are significant differences between the three termination codons. The UAA (ochre) codon has no known naturally occurring suppressor tRNAs. In the haemoglobin system, we have an assay for the presence of UAA readthrough but no activity is found in reticulocyte tRNA. Induced UAA suppressor tRNAs in prokaryotic systems are found to be detrimental causing a doubling of the generation time<sup>4</sup>. It is possible that efficient UAA suppressors might be lethal. However, cells with either UGA or UAG suppressor tRNAs appears to be healthy and indeed some readthrough proteins made by suppressing these two termination codons seem to be used in natural systems.



**Fig. 6** Further fractionation of fractions 5 to 8 of Fig. 5 after aminoacylation with tryptophan. Yeast synthetase was passed over a G-100 column to remove any remaining yeast tRNA. Fractions 5 to 8 from Fig. 5 were charged with  $^3\text{H}$ -Trp<sup>46</sup>. The reaction was stopped with the addition of 1/10 volume 2 M NaAc (pH 5.0) and 1 volume phenol saturated with 50 mM NaAc (pH 5.0). All operations were at 4 °C. The tRNA was precipitated, washed once with 66% EtOH and redissolved in 2 ml 0.4 M NaCl and loaded on to a 12-ml BD column<sup>22</sup>. The first half of fraction 1 represents the effluent from loading the sample. The last half of fraction 1 and fraction 2 were eluted with 0.4 M NaCl. Fractions 3 to 13 were eluted with 0.8 M NaCl and fractions 14 to 18 were eluted with 1.5 M NaCl, 20% EtOH. All column buffers contained 10 mM MgCl<sub>2</sub> and 50 mM NaAc (pH 5.0). 50  $\mu\text{l}$  of each fraction was TCA precipitated. The  $^3\text{H}$ -Trp tRNA<sup>Trp</sup> peak (not shown) was primarily in fraction 14 but also in fractions 15 to 17. None was detected before fraction 14. 8  $\mu\text{l}$  of each fraction was assayed for Trp acceptance activity<sup>46</sup> as shown in the graph. Fractions 14–15 and 16–17 were pooled before the assay. The fractions were then assayed for suppression activity as in Fig. 5. The film was exposed for 2 d.

have different meanings. Perhaps the codon UAA means that translation must 'always stop' and perhaps there are very few (or no) exceptions to this in normal cells. The codons UAG and UGA may mean 'usually stop'. However, naturally occurring tRNAs will occasionally read UAG or UGA, resulting in the production of natural readthrough proteins. In addition, double stop signals are used to ensure the complete termination of protein synthesis after a given codon and are abundant in prokaryotic systems<sup>31</sup>. A low level of natural suppressor would be unable to read through a double stop system at a significant frequency. The selective utilisation in mRNAs of the three different termination codons and multiple stop signals allows the systems to have complete stop signals as well as partial or controllable stop signals. Nature has thus achieved a measure of control of gene expression at the level of termination. Diversification and evolutionary experimentation can then be carried out; occasionally a peptide is added to the C terminus of a selected protein. The resulting readthrough protein may perform a unique and perhaps essential function.

We suggest that natural readthrough is used occasionally to create proteins in which two different polypeptide chains are joined together. This ability may be used in a selective fashion, perhaps to control key biological processes. It would be of interest, for example, to see whether suppressor tRNAs are synthesised at key points in cellular development or perhaps during oncogenic conversion. Development of this process has provided an opportunity for exploiting the effects of producing novel proteins by joining different segments.

Is it possible that this type of experimentation was found to be very useful in the evolution of biological systems? Perhaps natural readthrough was a precursor to a more complex system of synthesising proteins by bringing together different polypeptides: RNA splicing<sup>32</sup>. The power of the splicing system, in contrast to the readthrough system, is that the segments to be joined together need not be contiguous in the genome of the organism. Splicing is also far more efficient than readthrough. Nonetheless, the net result of splicing and natural readthrough may be quite similar. We suggest that the utility of using occasional readthrough proteins which join together different polypeptide segments may have some of the positive features which are present in the more sophisticated RNA splicing mechanism which plays a significant role in the expression of genetic information in eukaryotic cells.

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## Evolutionary implications of termination suppression

The difference in the way nature uses termination codons suggests a rationale for the existence of three termination codons in the genetic code. It is possible that termination codons

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## LETTERS

## Superclusters and galaxy formation

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Most galaxies and rich clusters of galaxies<sup>1</sup> are concentrated in superclusters, and there are great distances between superclusters<sup>2-6</sup>. In this paper we study the structure of superclusters using Zwicky clusters<sup>7</sup> as principal tracers of the large-scale structure of the Universe. Our study is restricted to the southern galactic hemisphere.

Using Huchra's compilation of available redshifts of galaxies we have found mean redshifts of Zwicky clusters of distance class 'near' in the region  $22 \text{ h} \leq \alpha \leq 4 \text{ h}$ . Of the 88 clusters in the area considered, redshifts have been measured for 56. The redshifts of the remaining 32 clusters have been estimated on the basis of luminosity function and apparent diameter. The distribution of clusters in the sky in two redshifts intervals is given in Fig. 1.

The prominent triangle-shaped aggregate seen in Fig. 1a is the Perseus supercluster. Its richest cluster A426<sup>1</sup>, is located at the northern tip of the supercluster. Further, rich clusters are situated in the western and southern tips—the Pegasus cluster U487<sup>8</sup> and A194, respectively. Other clusters form chains joining the tips of the supercluster. A chain is located close to the Milky Way, forming the northern boundary of the Andromeda supercluster.

In Fig. 1b two superclusters can be seen: the Pegasus supercluster in the right-hand section of the figure and the Cetus supercluster in the lower left-hand section. In the central area of Fig. 1b there are no clusters. This 'big hole' is surrounded by superclusters: the Perseus supercluster forms a wall at the near side of the hole, superclusters seen in Fig. 1b form walls at the left and right sides, and superclusters at redshifts  $10,000 \leq V_0 < 15,000 \text{ km s}^{-1}$  form walls at its far side.

The distribution of redshifts of galaxies in the central area of the Perseus supercluster (where there are no clusters) is given in Fig. 2a; Fig. 2b shows the distribution of redshifts of clusters of galaxies in the whole area of the Perseus supercluster, and Fig. 2c shows the distribution of redshifts of clusters in the remaining area outside the Perseus supercluster. There is a very strong peak in Fig. 2a and b at the redshift  $5,000 \text{ km s}^{-1}$  due to the Perseus supercluster. The distribution of redshifts at large redshift values is similar for galaxies and clusters. This suggests that galaxies situated in the central area of the Perseus supercluster form a thin stratum at the same distance as clusters form chains. The distribution of redshifts in Fig. 2c is completely different since it is caused by other superclusters situated at a greater distance from us.

In the direction of the Perseus supercluster there are only a few foreground and background galaxies, the vast majority of galaxies of magnitude  $13.0 < \text{mag} \leq 15.0$  belong to the Perseus supercluster. Thus we can use galaxy counts to estimate some numerical properties of the Perseus supercluster. The results are presented in Table 1.  $N$  denotes the number of Palomar Observatory Sky Survey fields,  $L$  the luminosity calculated by the method suggested by Jõeveer, Einasto and Tago<sup>3</sup>,  $B$  the surface brightness, and %S is the per cent of spiral galaxies in the respective area. Morphological types of galaxies were taken from Nilson<sup>8</sup>. In the right-hand column the number of radio galaxies is given<sup>9</sup>.

The data presented here indicate that the Perseus supercluster of galaxies is surrounded by chains of clusters of galaxies which join main density peaks—rich clusters. Main peaks have a low percentage of spiral galaxies (most galaxies are elliptical, or S0 galaxies) and contain many radio galaxies. In the central area of the supercluster the density of galaxies is small, there are no clusters here, and almost all the bright galaxies are spirals.

The total area covered by the Perseus supercluster is  $5,400 \text{ Mpc}^2$ , its mean thickness is 7 Mpc, volume  $3.8 \times 10^4 \text{ Mpc}^3$ .

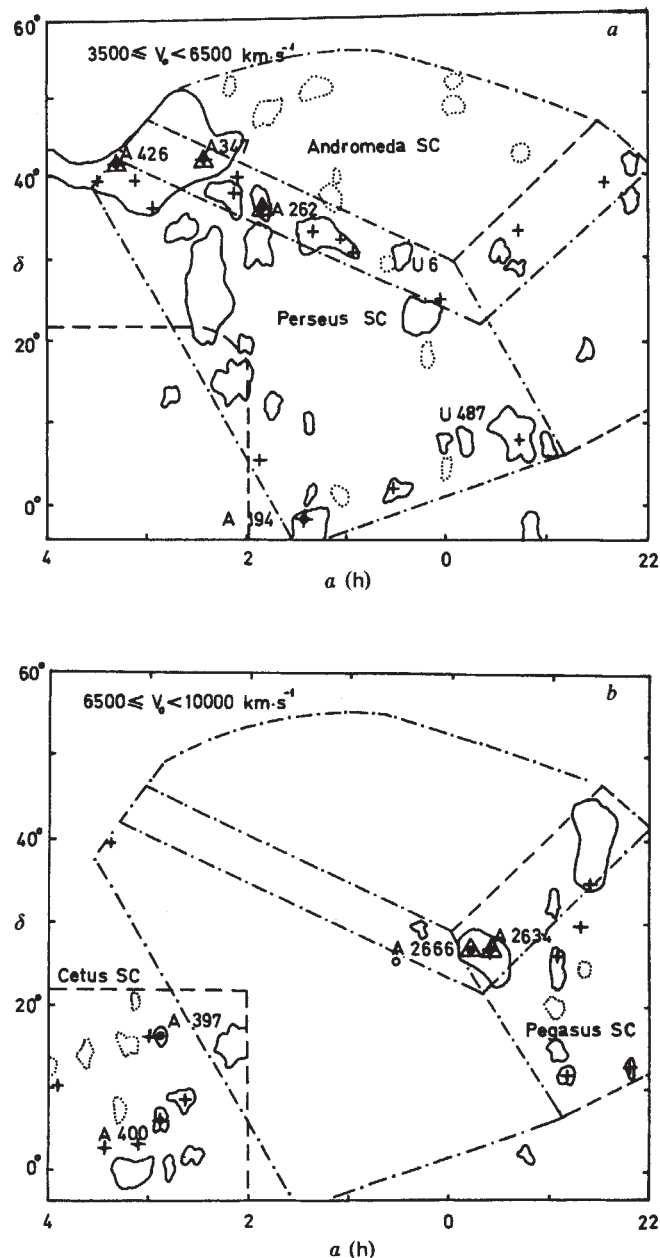
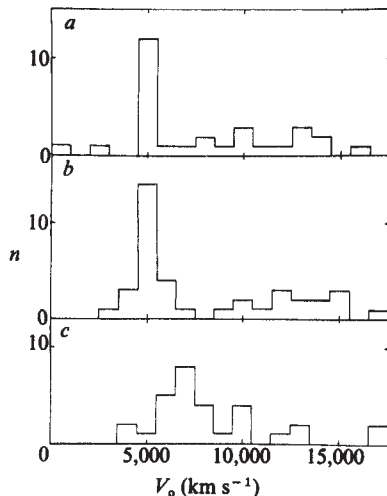


Fig. 1 The distribution of Zwicky clusters of redshifts  $3,500 \leq V_0 < 6,500 \text{ km s}^{-1}$  (a), and  $6,500 \leq V_0 < 10,000 \text{ km s}^{-1}$  (b). Zwicky clusters with measured redshifts have been drawn by solid lines, the clusters with estimated redshifts by dotted lines. Abell clusters are shown as filled circles, radio galaxies as crosses, and X-ray sources as triangles. Approximate boundaries of superclusters are shown as dashed and dotted-dashed lines.



**Fig. 2** The distribution of redshifts of galaxies in the central area of the Perseus supercluster (10 Palomar survey fields) (a), Zwicky clusters in the whole Perseus supercluster area (b), and Zwicky clusters outside the Perseus supercluster area (c).

and mass (adopted mean mass-to-luminosity ratio 150)  $2.7 \times 10^{16} M_{\odot}$ . The mean density in the supercluster is 10 times greater than the critical cosmological density<sup>6</sup>.

Neighbouring superclusters are in contact and contain common elements. For example, the chain of clusters between clusters A426 and U6 forms the northern boundary of the Perseus supercluster and the southern boundary of the Andromeda supercluster.

In the area under study in redshift interval  $3,500 \leq V_0 < 10,000 \text{ km s}^{-1}$  essentially all clusters are members of four superclusters (see Fig. 1). The space between superclusters (about 98% of the total volume studied<sup>6</sup> contains no clusters and very few bright galaxies.

A nonlinear theory of the growth of inhomogeneities suggests that the amount of matter concentrated in superclusters (initial positive density perturbations) is only a little larger than the total mass of the dark matter in big holes (initial negative density perturbations)<sup>6</sup>. If the density of matter associated with superclusters is  $\Omega_{sc} = 0.2$  (ref. 3), then the overall mean matter density is  $\Omega = 0.3$ . The different large-scale spatial distribution of dark and luminous matter cannot be reconciled with pure gravitational clustering theories<sup>10</sup>.

On the other hand, the morphology and structure of superclusters suggest that it is impossible to form superclusters in a wholly gaseous medium. In a gaseous proto-supercluster, the formation of galaxies has to begin in the central part of it, and would lead to a large cluster of galaxies rich in ellipticals in the centre of the supercluster<sup>11</sup>. Towards the edges the supercluster would be much thinner, and most galaxies in this region would be spirals. The observed structure is just the opposite.

**Table 1** Data on the Perseus supercluster

| Supercluster region | <i>N</i> | $\frac{L}{(10^{12} L_{\odot})}$ | $\frac{B}{(10^9 L_{\odot} \text{ Mpc}^{-2})}$ | %S        | No. of radio galaxies |
|---------------------|----------|---------------------------------|-----------------------------------------------|-----------|-----------------------|
| Mean peaks          | 3        | 30                              | 79                                            | 52        | 4                     |
| Cloud around A426   | 8        | 39                              | 39                                            | 58        | 5                     |
| Perseus chain       | 7        | 60                              | 68                                            | 68        | 6                     |
| Other chains        | 15       | 43                              | 23                                            | 79        | 2                     |
| Central area        | 10       | 9                               | 8                                             | $\geq 95$ | 0                     |
| Total               | 43       | 181                             | 33                                            | 68        | 17                    |

This difficulty can be avoided if, following Rees<sup>12,13</sup>, we suppose that the formation of galaxies was a two-stage process, but also that it involves much larger spatial dimensions than Rees suggested. In the first stage proto-superclusters and big holes had to form from the non-dissipative dark matter<sup>14</sup>. As demonstrated by numerical simulations non-dissipative matter would eventually form the observed structure with high density tips, intermediate density chains and low density walls, as long as the initial spectrum of perturbations contains large scale components<sup>15</sup>. In the second stage, hot gas, by cooling and settling down into the potential wells caused by dark matter, will form galaxies and clusters of galaxies<sup>16</sup>.

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## The source of the Jupiter S-bursts

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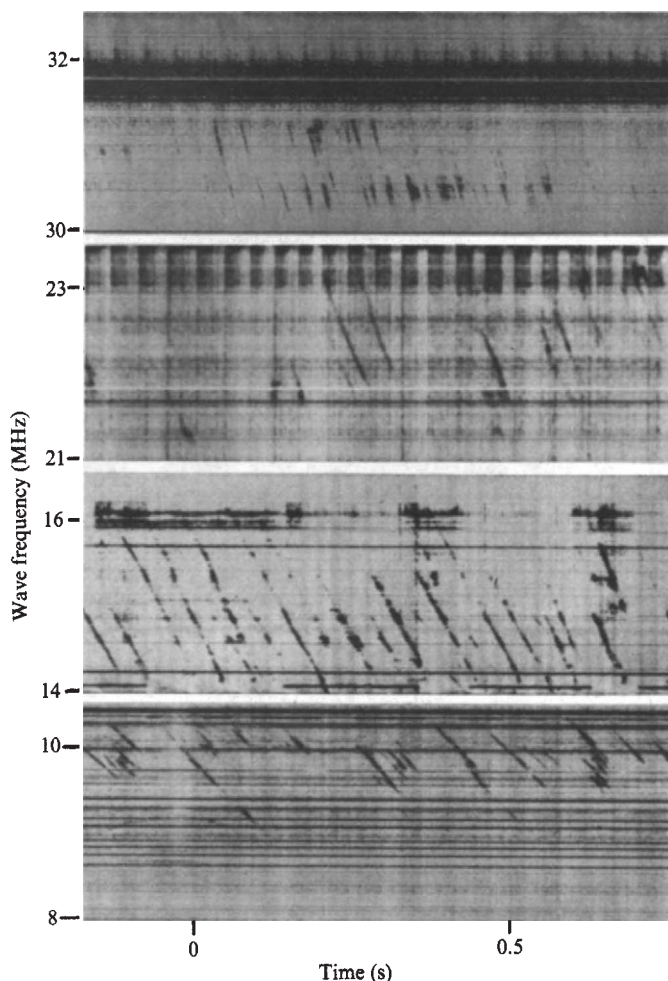
The transient radio S-bursts from Jupiter are strongly correlated with the orbital position of the satellite Io for all wave frequencies between 8 MHz and 30 MHz, and with system III longitude above about 15 MHz. The observations are most easily explained if the electrons generating the S-bursts are confined to the planetary magnetic flux tube intersecting Io (the IFT)<sup>1</sup>. The primary source of the electron energy is thought to be the electromotive force (EMF) generated by the relative motion of Io across the Jupiter magnetic field, the S-bursts being the result of convective instabilities in the consequent current flow in the IFT. The electrons are assumed to emit coherent electromagnetic radiation in the Doppler cyclotron mode as they travel up the IFT after being accelerated near its feet in the Jupiter ionosphere to energies of a few keV (refs 2-4).

Simple calculations using the observed rate of change of wave frequency with time for the S-bursts ( $df/dt \sim -10$  to  $-20 \text{ MHz s}^{-1}$ ) give 3-12 keV for the electron energy, and observations over a long period have always yielded electron energies in this range<sup>5</sup>. The narrow bandwidth of individual bursts ( $\Delta f \sim 10 \text{ kHz}$ )<sup>6</sup> implies that their velocity spread is very small ( $\Delta v/v \sim 10^{-3}$ ), so that the acceleration mechanism must be fairly stable and almost monoenergetic. At the same time, the magnetic field intensity in the IFT will vary as Jupiter rotates under Io owing to the non-centred location of the Jupiter



magnetic dipole<sup>7</sup>. Some variation in the dynamical properties of the electrons correlated with the longitude of Io, and consequently in the properties of the S-bursts, might hence be expected.

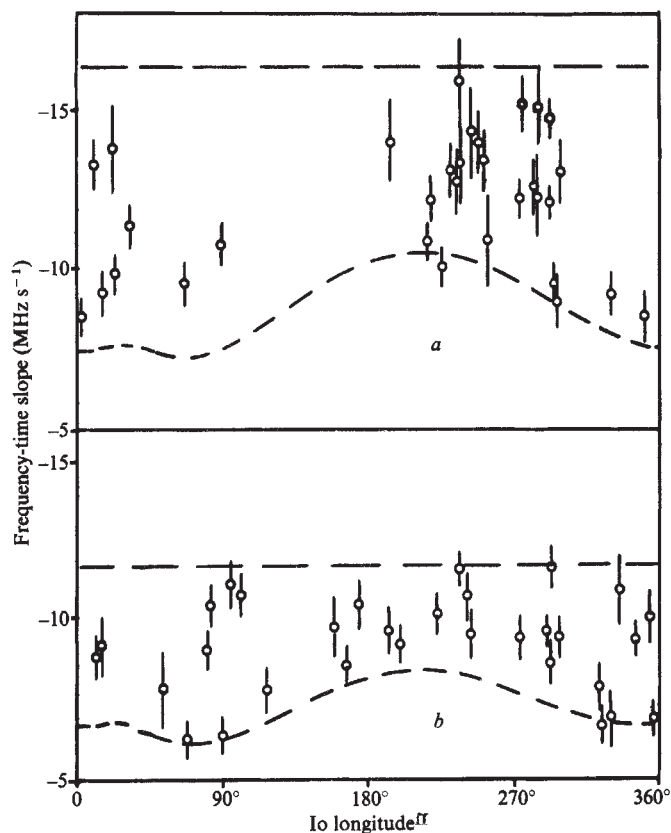
The S-bursts were observed between 1972 and 1979 in the frequency range 2.5–17 MHz with the 650 × 650 m Llanherne telescope, in the frequency 17–29 MHz with a 60 × 60 m array, and at 24–35 MHz using a new 85 × 170 m array of 4,096 dipoles. RF signals were recorded on video tape for analysis with a 256-channel spectrum analyser with frequency resolution 10 kHz and time resolution 600  $\mu$ s. Examples of S-burst spectra at 9, 16, 22 and 32 MHz are shown in Fig. 1. The observed distribution of  $df/dt$  with Io longitude for all the bursts observed



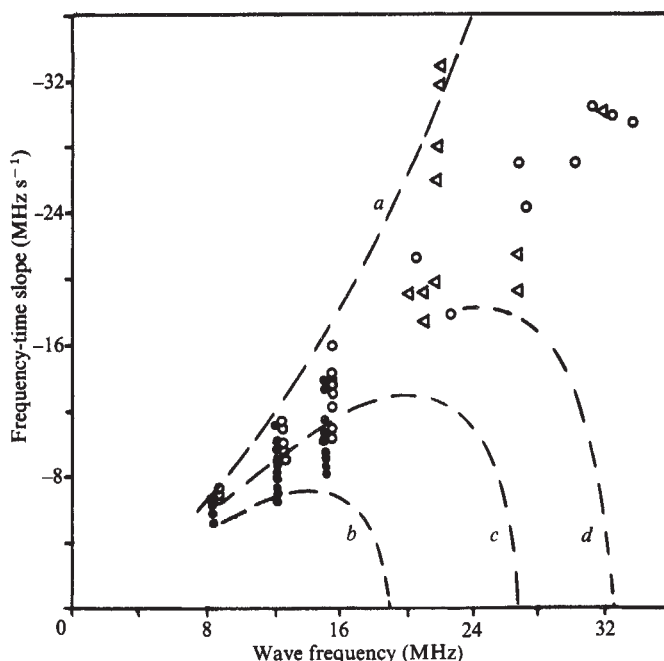
**Fig. 1** Dynamic spectra of S-bursts near wave frequencies of 9, 15, 22 and 32 MHz showing their increase in frequency-time slope with frequency.

near 12 and 15 MHz is shown in Fig. 2. Each point gives the mean value and standard deviation of  $df/dt$  during each 5-min daily observation period. Although  $df/dt$  varied between  $-6$  and  $-15 \text{ MHz s}^{-1}$  during the period of the observations, the variation on any particular occasion was much less, with the ratio of standard deviation to the mean being typically 0.1. That is, all the bursts, of the order of a thousand, observed in 5 min on a particular day had similar frequency-time slopes but varied considerably from day to day. In Fig. 3, the observations of  $df/dt$  at various wave frequencies between 5.5 and 35 MHz are shown alternatively as a function of frequency for two regions of system III (1965) sub-Io longitude  $L$  with  $180^\circ < L < 260^\circ$  and  $320^\circ < L < 040^\circ$ , chosen for high and low values of the magnetic field  $B_0$  at the foot of the IFT.

If it is assumed that for emission at a particular wave frequency, taken to be the cyclotron frequency, the electrons are



**Fig. 2** Observed variation of the  $df/dt$  of S-bursts with system III longitude of Io. *a*, Wave frequency 15 MHz. *b*, Wave frequency 12 MHz. In each section the upper and lower dashed lines show the limits expected for  $df/dt$  with electron energy 3 keV and initial pitch angles 0 and  $\pi/2$  respectively.



**Fig. 3** Observed variation of  $df/dt$  with wave frequency from 8.5 MHz to 34 MHz. ●, Present observations,  $320^\circ < L < 040^\circ$ ; △, observations reported by Desch, Flag and May<sup>3</sup>,  $180^\circ < L < 260^\circ$ ; ○, present observations  $180^\circ < L < 260^\circ$ . The dashed lines (*a*) and (*b*) show the calculated limits of  $df/dt$  for  $320^\circ < L < 040^\circ$ , and (*a*) and (*c*) for  $180^\circ < L < 260^\circ$ . The line (*d*) shows the lower limit of  $df/dt$  expected for northern hemisphere sources only ( $180^\circ < L < 260^\circ$ ). Electron energy, 3 keV.

all accelerated in the ionosphere to the same energy, then their longitudinal velocity at the point of S-burst emission will depend only on their initial pitch angle  $\phi_0$  and on  $B_0$ . That is,

$$\frac{df}{dt} = -KV_{\parallel} = -KV \left( 1 - \frac{B}{B_0} \sin^2 \phi_0 \right)^{1/2}$$

where  $K$  is constant and  $B$  is magnetic field at emission point. The maximum and minimum values of  $df/dt$ , obtained by taking  $\phi_0 = 0$  and  $\pi/2$ , will vary with wave frequency  $f(\propto B)$  and with  $B_0$  and hence  $I_0$  longitude. Figure 2 shows how the limits of  $df/dt$  calculated in this way vary with longitude for wave frequencies 12 MHz and 15 MHz. In Fig. 3, the same limits are shown as a function of wave frequency for the two restricted longitude regions specified earlier. Both northern and southern hemisphere values of  $B_0$  were used for wave frequencies below 17 MHz since polarisation observations have shown that in this frequency range S-bursts may have either right-hand or left-hand polarisation<sup>9</sup> and may presumably originate in either hemisphere. Above 17 MHz, northern hemisphere sources were assumed.

It can be seen that all the observed values of  $df/dt$  fit reasonably well between limits calculated using an electron energy of 3 keV whether they are plotted against  $I_0$  longitude or wave frequency. That is, with this model only a single value of electron energy with a range of initial pitch angles from 0 to  $\pi/2$  is needed to account for the observed wide variation of  $df/dt$ . The observations suggest that the electron acceleration is stable over long periods, a result which might be expected from the properties of the  $I_0$  unipolar inductor<sup>4</sup>.

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## Changes in the optical light curve of the Crab pulsar between 1970 and 1977

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**The pulsed optical radiation from the Crab pulsar has a characteristic and well determined profile. As the pulsar is comparatively young, the slope of this profile may be expected to change appreciably within only a few years. Our observations over an interval of 7 yr show that any such changes are surprisingly small, amounting to <1% of the peak pulse intensity.**

The two profiles of the pulsed optical radiation from the Crab pulsar were obtained as follows, in sufficiently similar circumstances to allow us to search for small differences. The first observations by Nelson *et al.*<sup>1</sup> were made at the Lick Observatory between 15 May 1969 and 3 May 1970 using the 0.9-m Crossley reflector. Figure 1 shows a composite of the data which were collected in 25- $\mu$ s intervals but shown here in 250- $\mu$ s intervals—in groups of 10 bins. The data cover 72% (23.8 ms) of the cycle. The second observation was made by Smith *et al.*<sup>2</sup> on 26 January 1977 with the 3.9-m Anglo-Australian telescope,

using an FW 130 photomultiplier with S20 response. These latter data were collected in 20- $\mu$ s intervals.

The spectral responses of the two sets of observations were similar, and although no special precautions were taken to avoid sensitivity to polarisation effects, we do not consider that these could seriously affect our results. Because of the similarity of the pulse profile at different wavelengths<sup>3</sup> any difference in spectral response would have to be enormous to affect the results.

After allowing for the lengthening of the period by 1 part in 350, the light curves are remarkably similar. Bearing in mind that the intensity zeros and the relative phases of the two curves are unknown, we decided to fit the main peaks as closely as possible and look for any remaining differences. We first adopted a time in the Smith data corresponding to the first interval in the Nelson data. For each Nelson bin the corresponding point in the time base of the Smith curve was found by multiplying the bin number by

$$\frac{25}{20} \times \frac{0.033\ 200\ 3858}{0.033\ 106\ 1538}$$

that is, by the product of the ratio of bin sizes and the ratio of the periods<sup>4</sup>. The Smith count corresponding to each Nelson bin was

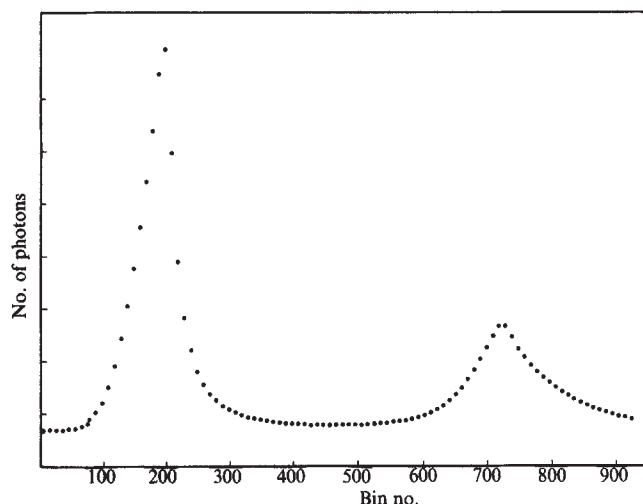


Fig. 1 Nelson *et al.*<sup>1</sup> light curves of the Crab Nebula pulsar. The original 25- $\mu$ s bins have been averaged in groups of 10.

then found by four-point lagrangian interpolation between the four Smith bins closest to the Nelson bin. It can easily be shown that, in the mean, this interpolation reduces the effective dispersion in the Smith observations by a factor 0.77. The Smith curve was then regressed on to the Nelson curve over  $\pm 6$  ms centred on the main pulse. The whole procedure was repeated for a series of time-offsets between the two light curves in search of the minimum r.m.s. fit. The differences between the two complete light curves after the best fit had been obtained on the main peak are shown in Fig. 2. The data in Figs 1 and 2 are plotted in bins 250  $\mu$ s wide, as we find that the differences are too small to show clearly in 25- $\mu$ s bins. The scatter of points in Fig. 2 is somewhat wider than would be expected from statistics alone.

The two main pulse profiles evidently have a very similar shape. There are, however, two outstanding points on the steepest part of the falling slope 0.4 ms after the peak (bins 211–220 and bins 221–230). Both these show a 1% discrepancy from the intensity at that part of the curve, which is well above the statistical uncertainty. Figure 3 shows the data from the individual 25- $\mu$ s bins in this region: the smoothness of the curve indicates that the difference may be real, representing a slower rate of fall in the later light curve.



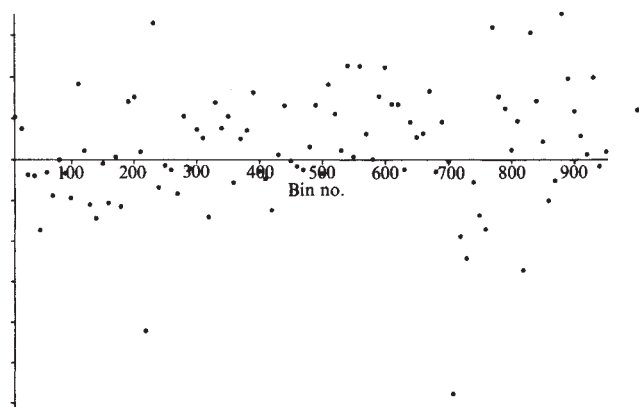


Fig. 2 The difference between the Smith *et al.*<sup>2</sup> curve and the 1970 curve. The ordinates have been averaged over 10 bins.

Apart from this small effect the shape of the main pulse has not changed significantly; the other differences between bins 0–300 are scattered fairly randomly about zero. No significant improvement in the two discrepant bins can be made without seriously worsening the fit elsewhere in the main pulse.

Using this best fit, three other differences can be seen in the rest of the light curve as follows: (1) base level changes: the low levels before the main pulse and the interpulse cannot both be fitted by our procedure. As the intensity is least before the main pulse<sup>2,5</sup>, we interpret the change in terms of a constant level at this point; the result is then an increase in the level between the main pulse and the interpulse. The increase is  $0.15 \pm 0.05$  (s.e.)% of the peak intensity above minimum, appearing between bins 500 and 700.

(2) Relative intensity of pulses: the low values between bins 710 and 760 represent a decrease of interpulse intensity of  $0.91 \pm 0.25$  (s.e.)%.

(3) Spacing of pulses: the low values are not uniformly distributed around the peak of the interpulse. The interpretation of this is uncertain, as there is an irregular spread of comparatively large difference values in the region of the interpulse. A better fit for the interpulse is obtained if the spacing between main pulse and interpulse is increased by  $15 \mu\text{s}$ , but it is doubtful whether this is significant. Any relative movement of the pulses cannot be greater than  $\sim 25 \mu\text{s}$ , corresponding to about  $0.25^\circ$  of pulsar rotation phase.

An interval of 7 yr in the life of the Crab pulsar, which is  $<1,000$  yr old, might be expected to produce appreciable changes in the light curve. We find that the only significant changes amount to  $<1\%$  of the peak intensity: they appear as a small change in the steep fall-off from the main peak, a change in

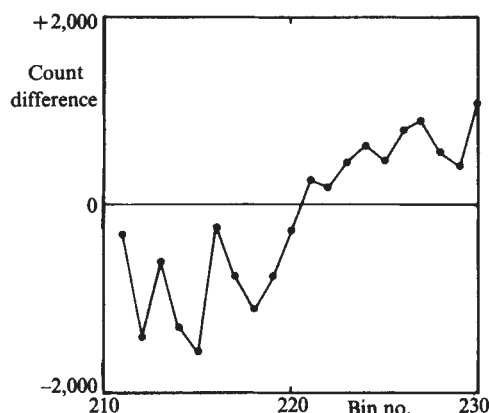


Fig. 3 Differences for 25- $\mu\text{s}$  bin numbers 210–230, showing a change in the falling portion of the main pulse.

the relative levels of the baseline before and after the main peak, and a decrease in the interpulse intensity relative to the main peak.

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## The frictional anisotropy of diamond

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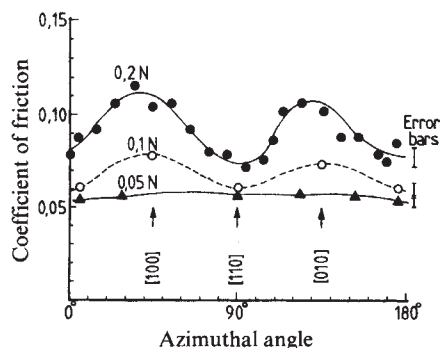
If a diamond stylus is slid over a flat diamond surface in air the friction may depend significantly on the direction of sliding. The most marked effect is on the {100} faces. Along the  $\langle 100 \rangle$  direction the friction is high (coefficient of friction  $\mu \approx 0.15$ ) while along the  $\langle 110 \rangle$  direction it is low ( $\mu \approx 0.07$ ). This has been variously attributed to surface roughness (Coulomb friction) or to processes involving surface adhesion. (Some of these processes and related ideas are discussed in ref. 1.) We describe here some recent experiments which show that frictional anisotropy occurs only when the contact pressure exceeds a critical value. In these conditions cathodoluminescence studies of the diamond flat indicate that the frictional anisotropy is largely due to surface and sub-surface damage produced in preferred crystallographic directions by the sliding process itself. However, the results do not, as yet, provide a detailed account of the mechanism of energy dissipation.

The experiments were carried out with a very simple apparatus and involved the sliding of diamond styli over the polished (001) surface of Type I and Type II diamond. The flat surface was cleaned in air with solvents so that it was covered with oxygen and possibly other adsorbed contaminants. The styli were hemispherical sliders, of radius of curvature 18, 80 and  $140 \mu\text{m}$ . The normal load could be varied from 4.9 mN (0.5 g) to 19.6 N (2,000 g) and the sliding speed was  $0.2 \text{ mm s}^{-1}$ .

The Type I diamond was polished to within  $1^\circ$  of a (001) plane. Polishing lines, parallel to the  $\langle 100 \rangle$  direction, were visible in the optical microscope and about  $1 \mu\text{m}$  apart. The Type II diamond was several degrees off the (001) plane and the polishing lines were somewhat finer. Both diamonds showed characteristic frictional anisotropy the lowest friction being along the  $\langle 110 \rangle$  directions and the highest along the  $\langle 100 \rangle$  directions. With the Type I diamond the frictional anisotropy was not quite symmetrical, the friction in the [010] direction being higher than in the [100]. The reasons for this will be discussed in a later, more detailed publication. With Type II diamond the frictional anisotropy was symmetrical as shown in Fig. 1. It is seen that at a load of 0.2 N (20 g) the coefficient of friction in the  $\langle 100 \rangle$  directions is about 0.12 and about 0.07 in the  $\langle 110 \rangle$  directions. This is not a Coulomb type of behaviour where the friction is attributed to the climbing of one surface over asperities on the other, for in such a situation the coefficient of friction is determined solely by the surface topography and is independent of the load. In contrast, in the present experiments, if the load is reduced to 0.05 N (5 g) the anisotropy disappears. This suggests that the frictional anisotropy is associated with damage produced in the flat diamond surface when the contact pressure exceeds some critical value discussed below.

This was confirmed by experiments carried out on the polished (001) surface of a Type II diamond using a stylus of radius  $R = 140 \mu\text{m}$  and loads ranging from about 4.9 mN (0.5 g)

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**Fig. 1** Coefficient of friction  $\mu$  of a diamond stylus (radius of curvature  $18\text{ }\mu\text{m}$ ) sliding over the (001) face of a Type II diamond. At a load of  $0.2\text{ N}$  ( $20\text{ g}$ ) the friction along the  $[100]$  direction is nearly twice the friction along the  $[110]$  direction. The frictional anisotropy is not due to a Coulomb type of friction since if the load is reduced to  $0.05\text{ N}$  ( $5\text{ g}$ ) the anisotropy disappears. Similar effects were also observed on the Type I diamond.

to  $19.6\text{ N}$  ( $2,000\text{ g}$ ). At low loads the friction ( $\mu \approx 0.07$  to  $0.08$ ) was essentially the same in the  $[100]$  and  $[110]$  directions and did not increase appreciably with load. However, in the  $[100]$  direction there was a sudden rise in friction when the load exceeded a critical value  $W_c$ : for example, at a load of  $11.8\text{ N}$  ( $1,200\text{ g}$ )  $\mu$  reached a value of  $0.14$ . The tracks formed in this direction of sliding were examined by cathodoluminescence which reveals surface and subsurface damage. Figure 2 shows that as the damage increases, with increasing load, the friction increases. (Note that a load  $W = 6.9\text{ N}$  where there is a slight diminution in the amount of damage the friction shows a small drop).

A study was also made of the critical load  $W_c$  at which a marked rise in friction in the  $[100]$  direction occurred as a function of the radius of curvature  $R$  of the slider. Our experiments showed that  $W_c$  was closely proportional to  $R^2$ . If we assumed that this transition is associated with the generation of appreciable subsurface damage we may apply the principles of fracture mechanics. Frank and Lawn's<sup>2</sup> work on the growth of cracks in brittle solids shows that for static contact  $W_c$  should be proportional to  $R$  (Auerbach's law). On the other hand Lawn<sup>3</sup> has shown that in sliding contact for  $\mu > 0.02$   $W_c$  should be proportional to  $R^2$ . Our results therefore qualitatively agree with Lawn's theory and quantitatively agree if it is assumed that there are initial cracks in the surface of the order of  $0.5\text{ }\mu\text{m}$  deep. The proportionality between  $W_c$  and  $R^2$  implies that for elastic (hertzian) contact, the friction transition occurs when the mean

contact pressure reaches a critical value: in our experiments this has a value of order  $2,000\text{ kg mm}^{-2}$ .

We conclude that the frictional anisotropy is, to a large extent, due to surface and subsurface damage produced in preferred crystallographic directions by the sliding process itself. It is possible that some plastic grooving occurs in the friction track though the pressure of  $2,000\text{ kg mm}^{-2}$  seems too small for this. On the other hand some microplastic deformation may be involved and contribute to the energy dissipation but the major factor apparently involves subsurface cracking which is generated when the contact pressure exceeds some critical value. This is consistent with our observations that with softer styli such as steel or even tungsten carbide frictional anisotropy is not observed over an extremely wide load range. It is also consistent with the practical observation that in the polishing of diamond with diamond powder the polishing rate is very much higher in the directions of high friction presumably because of the increased ease of producing cracks in and beneath the surface. Although these results explain most of the relevant phenomena they do not yet provide a detailed account of the way in which the energy is dissipated.

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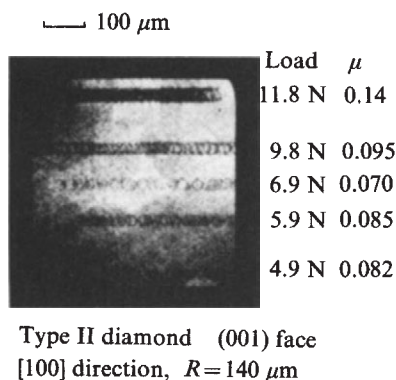
## Observations on internal structures of chrome alum dehydration nuclei

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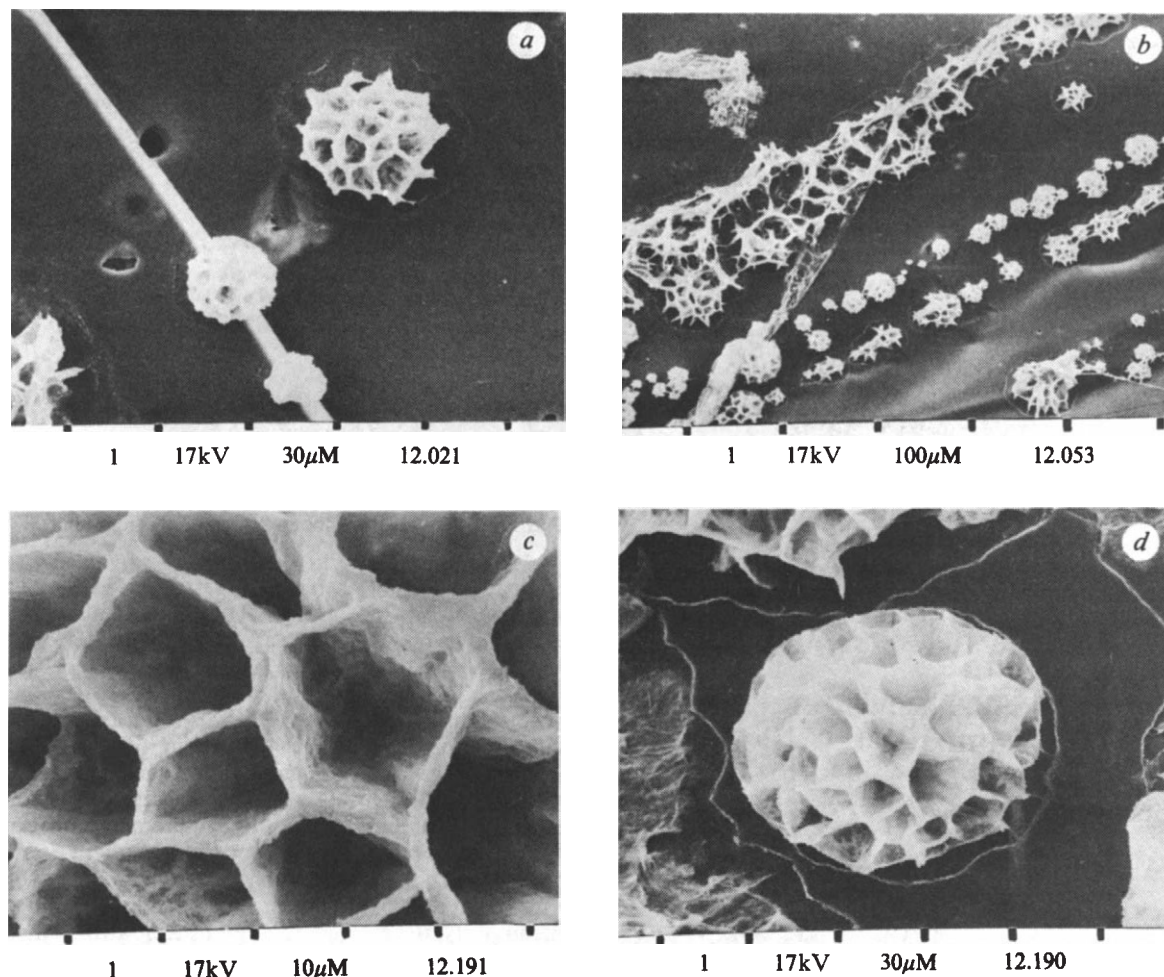


**Fig. 2** Cathodoluminescence observed on the (001) face of a Type II diamond after a single to-and-fro traversal with a diamond stylus of radius  $R = 140\text{ }\mu\text{m}$ . The corresponding loads and coefficients of friction  $\mu$  are indicated. There is a marked increase in subsurface damage and in  $\mu$  at a load of  $11.8\text{ N}$  ( $1,200\text{ g}$ ). Scale bar,  $100\text{ }\mu\text{m}$ .

Decomposition reactions of many solids proceed by nucleation and growth processes<sup>1</sup>, so that the kinetic expressions used<sup>2</sup> and the interpretation of rate data<sup>3</sup> are based on quantitative consideration of the progressive changes in geometry of advancing reactant/product interfaces. Conclusions from such kinetic analyses are often confirmed by optical microscopic observations. We describe here a method whereby information may be obtained, in favourable systems, about the three-dimensional shapes of nuclei and the disposition of product particles in the wake of interface advance. For the reaction described below, the dehydration of  $\text{KCr}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ , it is concluded that product shrinkage results in crack development through which water is evolved and crystallographic factors exert little control on the directions of interface advance during growth of the approximately hemispherical nuclei.

Most previous microscopic studies of solid state decompositions have been restricted to measurements of the numbers, distributions, sizes and shapes of nuclei within the two-dimensional plane that constitutes the crystal boundary surface. Such information is often an incomplete representation of the nuclei which may be three-dimensional assemblages of large numbers of small product particles. A new technique is described here whereby, in suitable systems, information can be obtained on





**Fig. 1** Scanning electron micrographs of replicas of nuclei developed on cleavage surfaces of chrome alum, partially dehydrated in vacuum, prepared by techniques described in the text. Magnification as shown by scale on photographs. *c* Is an area of *d* more highly magnified.

the texture of the product phase, including particle sizes and any topotactic relationship with the reactant. Such evidence is particularly valuable in the formulation of mechanisms of bond and lattice rearrangement steps within the restricted zone that is regarded as constituting the active reaction interface. A knowledge of crack dimensions may permit estimation of the impedance offered to volatile product escape during reversible reactions. Present mechanisms of interface processes are usually inferred from indirect evidence such as Arrhenius parameters<sup>1</sup>.

Figure 1 gives evidence of a regular internal crack development in the characteristic nuclei evolved during the vacuum dehydration of chrome alum ( $\text{KCr}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O} \rightarrow \text{KCr}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O} + 6\text{H}_2\text{O}$ ; this reaction was previously studied by Garner *et al.*<sup>4</sup>). These are scanning electron micrographs of replicas of the nuclei, which are apparently 'seen' from a viewpoint inside the crystal. The solid replication material fills the voids within the product assemblage that constitutes the nucleus. These photographs were obtained as follows. Large (~5 mm edge) solution-grown chrome alum crystals were cleaved by a sharp blow with a knife edge and acceptable planar surfaces were dehydrated 10–30 min in vacuum ( $\sim 10^{-4}$  torr) at ~300 K. The development of nuclei was confirmed by optical microscopic examination and, with minimum exposure to the atmosphere, the crystal was withdrawn from the vacuum and a drop of 2% solution of polyvinyl formal solution in dry chloroform carefully placed only on the surface of interest. After solvent evaporation and hardening of the replica (>24 h), the alum crystal was removed by dissolution in distilled water, the replica washed, dried and coated with gold–palladium before examination in the Stereoscan S180. (Alum crystals are not

sufficiently stable to be directly examined in the microscope.)

Figure 1 shows representative features of typical nuclei on surfaces present after partial dehydration of cleavage surfaces of chrome alum. Figure 1a includes a typical nucleus having a divergent distribution of laminar cracks between product assemblages of approximately hexagonal section. At the crystal boundary plane it is possible to identify the approximately circular outline of the nucleus, locally curving outwards in the vicinity of advancing cracks, that is seen by optical examination of the crystal surface<sup>1,4</sup>. The oblique line is a surface step or crack, at which two nuclei have been preferentially generated, though along much of this zone and at other sites of imperfection reaction has not, as yet, been initiated. Figure 1b shows that there are strongly preferred directions of alignment of nuclei. Nuclei in the upper aligned band have overlapped with interlinking of volatile product escape channels. The crack systems in the lower two parallel lines of nuclei have started interpenetration but overlap is, as yet, less extensive. The crack systems characteristic of a developed individual nucleus are shown at two magnifications in Fig. 1c and d.

The development of strain during dehydration<sup>5</sup> is believed to result in the generation of this internal regular hexagonal disposition of cracks within nuclei, which is somewhat similar to that found in columnar basalt<sup>6</sup> where it has also been ascribed to shrinkage. Crystallographic factors are of little significance in controlling the direction of interface advance during growth of hemispherical nuclei in this alum.

We suggest that examination of the internal structures of nuclei, as revealed by this replication technique, provides a new approach to the elucidation of the mechanisms of solid state

reactions. Evidence from the structures of dehydration nuclei developed in  $\text{KCr}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  supports earlier suggestions<sup>5</sup> that stress is an important factor in controlling product formation: a more detailed consideration of this and related reactions will be published elsewhere.

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## Palaeointensity and thermoluminescence measurements on Cretan kilns from 1300 to 2000 BC

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The strength of the geomagnetic field in Crete during Minoan times has been determined using kiln wall samples from three Minoan palaces, Faistos, Aghia Triada and Kato Zakros, from the Minoan village at Stylos Hania and a Roman kiln at Kalo Horio. The samples have been dated by thermoluminescence measurements providing absolute dates for the construction of a palaeointensity curve. Without the thermoluminescence dates it would have been necessary to use the archaeologists' estimates of the kiln ages, one of which is shown here to be in error by 500 yr.

Kiln material was used because: (1) pottery was not readily available; (2) it was hoped that both palaeointensity and palaeodirection results could be obtained; and (3) the last firing events which were related to the destruction of the Minoan palaces could be dated by thermoluminescence measurements.

The samples, from the kiln floor, walls and heat channels, consist of a fired calcareous mud, varying in appearance from the extremes of a well fired vesicular slag (Aghia Triada and site (1) Faistos) to orange, porous, friable, less well-fired, samples with a fine mud matrix containing fragments of shells, rocks and pottery (site 1 of Kato Zakros, Stylos Hania and site 3 of Faistos); the remaining samples (Kalo Horio and site 1 of Faistos) contain a mixture of both types. Kato Zakros (2) is a brick fragment and Kato Zakros (3), a tile fragment.

The palaces sampled were of middle and late Minoan age. The earliest palace site sampled was Faistos. Kiln FS (3) belongs to the first palatial period (~2000–1700 BC) which ended when the palace was destroyed by earthquakes in about 1730 BC (ref. 1). The palace was subsequently rebuilt but in about 1500 BC it was destroyed by earthquakes or, according to some authors, by the effects of a volcanic eruption of Santorini (refs 2–5). Kiln FS (1) belongs to this second palatial period. The palace of Aghia Triada was built to replace the second palace at Faistos and probably lasted to 1400 BC (ref. 6).

The palace at Kato Zakros is thought to date from 1700 to 1400 BC. Platon (personal communication) believes that it was

Table 1 Thermoluminescence ages (yr BP)

| Site         | Age of sample | Estimated % error | Average site age | Estimated % error |
|--------------|---------------|-------------------|------------------|-------------------|
| Stylos Hania | 3,882         | 6                 | 3,828            | 4                 |
|              | 3,840         | 6                 |                  |                   |
|              | 3,763         | 7                 |                  |                   |
| Faistos (1)  | 3,466         | 6                 | 3,466            | 6                 |
|              | 3,806         | 5                 |                  |                   |
| Faistos (3)  | 3,762         | 6                 | 3,784            | 4                 |
|              | 3,545         | 6                 |                  |                   |
| Aghia Triada | 3,472         | 5                 | 3,508            | 4                 |
|              | 3,370*        | 5                 |                  |                   |
| Kato Zakros  | 3,199*        | 5                 | 3,303            | 2                 |
|              | 3,139*        | 6                 |                  |                   |
|              | 3,289         | 5                 |                  |                   |
|              | 3,519         | 5                 |                  |                   |
| Kalo Horio   | 2,126         | 7                 | 1,960            | 5                 |
|              | 1,795         | 7                 |                  |                   |

\* These samples are tiles, all other samples are from kilns.

not rebuilt or inhabited after the catastrophic earthquake which occurred between 1450 and 1400 BC. The kiln at Stylos Hania is believed to be of late Minoan age<sup>7</sup> (~1300 BC); no pottery was found in the kiln but the nearby village is of that age. Kalo Horio, a Roman kiln was believed to date from ~50 BC.

The thermoluminescence (TL) method used was the quartz inclusion technique described by Fleming<sup>8</sup>. TL dates can only be produced from well-fired materials so the number of dates produced was limited because of vitrification or weathering of the samples. Sample preparation and the determination of the total archaeological dose is described elsewhere<sup>9</sup>. The crystal sensitivity change due to heating to 500°C varied between 5 and 40%. The final total dose error was that derived from the 'dose plateau' tests, the average total doses from two or three peaks only being taken when there was good agreement between them. The  $\beta$  dose rates were measured in two ways, using thermoluminescent dosimetry and also using an  $\alpha$  counting and potassium content method. The evaluation of environmental  $\gamma$  radiation was made on site by a portable NaI (T1) scintillometer. Results are presented in Table 1.

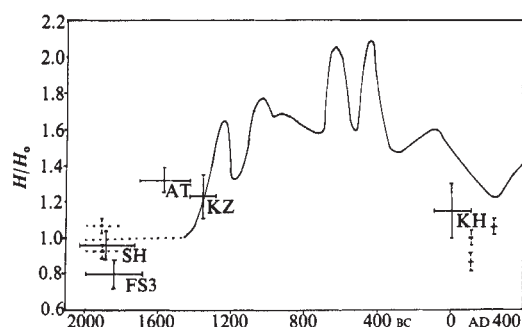
One disadvantage of using kiln material in palaeointensity work is that it was not originally selected by a potter and so is more inhomogeneous and complex mineralogically than pottery. Assessment of the mineralogical state of kiln wall material is complicated by the possibility that it has been repeatedly fired and cooled in various gaseous environments.

Attempts were made to determine the palaeointensities by two methods, that described by Shaw<sup>10</sup> and a modified Thellier method<sup>11</sup>. Alteration of one sort or another occurred in all samples used in Shaw's method. For most of the samples <10% of the NRM remained after demagnetisation at the peak a.c. field of 900 Oe. The ARM2 versus ARM1 gradients were generally <1, except for samples from Aghia Triada, in many cases they had several linear portions of differing gradients.

Table 2 Results using Shaw's method

| Site         | No. of results | Range of uncorrected palaeointensities (Oe) | Range of corrected palaeointensities (Oe) | Mean and error of palaeointensities (Oe) |
|--------------|----------------|---------------------------------------------|-------------------------------------------|------------------------------------------|
| Aghia Triada | 3              | 0.369–0.497                                 | 0.560–0.590                               | 0.580 ± 0.034                            |
| Stylos Hania | 5              | 0.572–0.422                                 | 0.365–0.479                               | 0.423 ± 0.035                            |
| Faistos (1)  | 3              | 0.259–0.786                                 | 0.249–0.519                               | ?                                        |
| Faistos (3)  | 4              | 0.776–0.330                                 | 0.395–0.333                               | 0.351 ± 0.037                            |
| Kato Zakros  | 5              | 0.499–0.791                                 | 0.474–0.557                               | 0.542 ± 0.058                            |
| (1)          |                |                                             |                                           |                                          |
| Kato Zakros  | 1              | ARM and TRM plots nonlinear                 |                                           |                                          |
| (2)          |                |                                             |                                           |                                          |
| Kato Zakros  | 3              | 0.485–0.788                                 | 0.512–0.560                               | 0.540 ± 0.048                            |
| (3)          |                |                                             |                                           |                                          |
| Kalo Horio   | 7              | 0.581–0.887                                 | 0.416–0.645                               | 0.505 ± 0.066                            |





**Fig. 1** The change in the ratio of the ancient geomagnetic field intensity  $H$  to the present day value,  $H_0$  (0.44 Oe) in Crete. Each point represents several palaeointensity determinations on kiln wall material from one site. The errors are the combined errors from all determinations. The curve produced by Walton<sup>13</sup> is shown for comparison. The Cretan points are labelled, AT, Aghia Triada; KZ, Kato Zakros; SH, Stylos Hania; FS3, Faistos site 3; KH, Kalo Horio. Walton's points which lie away from the smoothed curve are shown by dotted lines.

The TRM versus NRM graphs often showed similar features to the ARM2 versus ARM1 graphs, and where this was the case the correction suggested by Kono<sup>12</sup> was applied to portions of the graph with more than six points. The scatter of results was greatly reduced and for most sites the results were self consistent (Table 2). This method does not always yield reliable results<sup>12</sup> so it was checked using Coe's modified version of Thelliers' method<sup>11</sup>. Alteration of the samples during heating was again a problem and most sites showed a wide scatter of results. The only sites for which useable results were obtained, Kalo Horio ( $0.506 \pm 0.110$  Oe) and Kato Zakros 1 ( $0.484 \pm 0.082$  Oe), back up the results obtained using Shaw's method with Kono's correction.

The thermoluminescence results agree with the archaeologists' preferred chronology for the major sites. The result obtained for the less well known site of Stylos Hania suggests that it is older than was previously thought. The thermoluminescence results reinforce the suggested absolute chronology of the Minoan period but cannot rival the detail of relative chronologies deduced by the archaeologists.

The palaeointensity results in Table 2 are compared with those of Walton in Fig. 1. The age error bars on the palaeointensity curve are naturally larger than those of Walton's curve for which the relative ages of the samples are well known. It is unlikely that so precise a curve as Walton's can be extended further back in time, either using archaeological dating or absolute dating which is not yet capable of yielding the necessary precision.

Crete and Athens are  $\sim 3^\circ$  latitude apart; differences in geomagnetic field intensity in the two areas are therefore to be expected. The present value for this difference is 0.01 Oe which is smaller than the errors on the palaeointensity determinations. The maximum difference in geomagnetic field intensity, at present, for sites located at the latitudes of Crete and Athens is 0.03 Oe, so it is to be expected that the palaeointensity results for Crete and Athens will agree within experimental error. This is the case for Stylos Hania and Kato Zakros. The Kalo Horio datum falls at a time where Walton seems to have ignored some data points when constructing his palaeointensity curve. If the Kalo Horio result is to be compatible with Walton data then it is necessary to postulate a fairly rapid change in the geomagnetic field strength.

The palaeointensity results reinforce the thermoluminescence date obtained for Stylos Hania, a palaeointensity of 0.42 Oe being unlikely for a sample aged 1250 BC.

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## First measurements of gas phase sulphuric acid in the stratosphere

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Sulphuric acid is thought to be the most important nucleating agent in the stratosphere and thus has a key role in the formation of the stratospheric aerosol layer ('Junge layer')<sup>1,2</sup>. As the latter at least temporarily influences the Earth's radiation budget and thereby the Earth's climate<sup>3–5</sup>, sulphuric acid is a very important stratospheric trace gas. The formation of stratospheric sulphuric acid is considered to proceed via a not yet fully determined photoreaction chain, starting from  $\text{SO}_2$  (ref. 6). Sulphur dioxide, in turn, is thought to result from COS and possibly also from  $\text{CS}_2$  being transported from the troposphere into the stratosphere<sup>7</sup>. Direct  $\text{SO}_2$  injection is also possible. We report here the first measurement of gas phase sulphuric acid abundances in the stratosphere. Our method relies on a recent composition measurement of stratospheric negative ions which revealed the presence of species containing sulphuric acid molecules attached to  $\text{HSO}_4^-$  core ions<sup>8,9</sup>. Studies of relevant negative ion reactions at the NOAA-Aeronomy Laboratory<sup>10</sup> have confirmed the identifications and reactions of the observed ions, giving a firm basis for determination of stratospheric  $\text{H}_2\text{SO}_4$  abundances from negative ion composition measurements.

The first composition measurement of stratospheric negative ions showing the major species and their fractional abundances are given in Table 1. The experiment was performed at an altitude of 36.5 km over southwestern France on 10 November 1977. A reaction scheme including the major ions (Fig. 1) suggests that the new cores of the  $\text{HSO}_4^- (\text{H}_2\text{SO}_4)_n (\text{HNO}_3)_m$  ions are formed by  $\text{H}_2\text{SO}_4$  reactions (reactions (1) and (4) involving  $\text{NO}_3^- \text{HNO}_3$  and  $\text{NO}_3^- (\text{HNO}_3)_2$ ). Subsequent  $\text{H}_2\text{SO}_4$ -switching reactions lead to a replacement of  $\text{HNO}_3$  ligands by  $\text{H}_2\text{SO}_4$ . While the rate constants of the reactions (1) and (4), have been measured in the laboratory ( $k_1 = 8.6 \times 10^{-10} \text{ cm}^3 \text{ s}^{-1}$ ;  $k_4 = 4 \times 10^{-10} \text{ cm}^3 \text{ s}^{-1}$ )<sup>10</sup>, those of the switching-processes have not yet been measured but may safely be assumed to range around  $10^{-9} \text{ cm}^3 \text{ s}^{-1}$  with an uncertainty of a factor of  $\pm 2$ . Recombination processes of negative ions with positive ions which are not included in Fig. 1 probably all have rate constants  $\alpha$  around  $10^{-7} \text{ cm}^3 \text{ s}^{-1}$  at 36.5 km (ref. 11).

Considering the above rate constants the unknown sulphuric acid number density  $n(\text{H}_2\text{SO}_4)$  can be derived from the measured negative ion composition and the measured total positive ion number density  $n_+$  as follows: according to Fig. 1  $\text{NO}_3^- (\text{HNO}_3)_2$ , the most abundant member of the  $\text{NO}_3^- (\text{HNO}_3)_n$  family, is converted via reaction (4) to  $\text{HSO}_4^- (\text{HNO}_3)_2$  which, in

**Table 1** Mass numbers, tentative identifications and fractional abundances of the major negative ions observed at 36.5 km altitude

| Mass (AMU) | Ion                                                                           | Abundance (%) |
|------------|-------------------------------------------------------------------------------|---------------|
| 125 ± 2    | NO <sub>3</sub> <sup>-</sup> HNO <sub>3</sub>                                 | 2.6           |
| 161 ± 2    | HSO <sub>4</sub> <sup>-</sup> HNO <sub>3</sub>                                | 5.3           |
| 188 ± 2    | NO <sub>3</sub> <sup>-</sup> (HNO <sub>3</sub> ) <sub>2</sub>                 | 65.6          |
| 197 ± 3    | HSO <sub>4</sub> <sup>-</sup> H <sub>2</sub> SO <sub>4</sub>                  | 6.6           |
| 224 ± 3    | HSO <sub>4</sub> <sup>-</sup> (HNO <sub>3</sub> ) <sub>2</sub>                | 14.2          |
| 260 ± 3    | HSO <sub>4</sub> <sup>-</sup> H <sub>2</sub> SO <sub>4</sub> HNO <sub>3</sub> | 3.1           |
| 294 ± 3    | HSO <sub>4</sub> <sup>-</sup> (H <sub>2</sub> SO <sub>4</sub> ) <sub>2</sub>  | 2.6           |

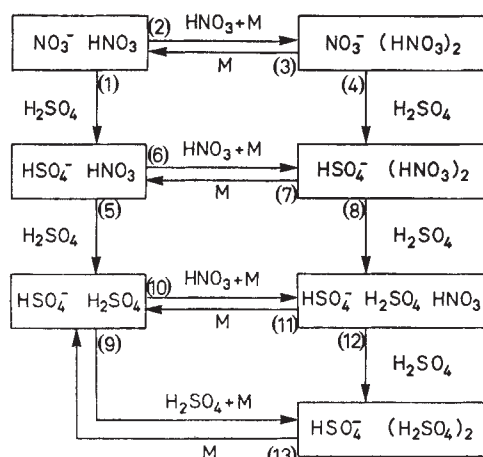
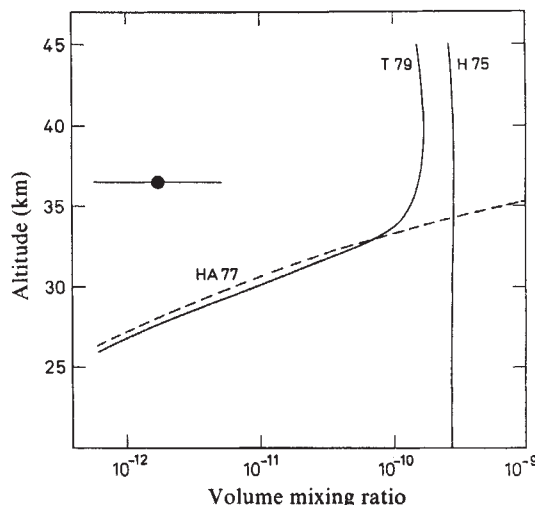
turn, is preferably converted to HSO<sub>4</sub><sup>-</sup>H<sub>2</sub>SO<sub>4</sub>HNO<sub>3</sub> (reaction(8)) and ultimately to HSO<sub>4</sub><sup>-</sup>(H<sub>2</sub>SO<sub>4</sub>)<sub>2</sub> (reaction (12)). Neglecting the thermal decomposition reactions (7), (11) and (13) the steady state continuity equation for the total number density  $n_s$  of the product ions HSO<sub>4</sub><sup>-</sup>(HNO<sub>3</sub>)<sub>2</sub>, HSO<sub>4</sub><sup>-</sup>H<sub>2</sub>SO<sub>4</sub>HNO<sub>3</sub> and HSO<sub>4</sub><sup>-</sup>(H<sub>2</sub>SO<sub>4</sub>)<sub>2</sub> becomes

$$(\text{NO}_3^-(\text{HNO}_3)_2)k_4n(\text{H}_2\text{SO}_4) = n_s\alpha n_+ \quad (1)$$

taking a measured  $n_+$  of  $2 \times 10^3 \text{ cm}^{-3}$  (ref. 9) which is consistent with a galactic cosmic ray ionisation source and the above  $\alpha$  the sulphuric acid number density can be derived from the negative ion abundances (Table 1) using equation (1). The resulting  $n(\text{H}_2\text{SO}_4)$  value is  $1.6 \times 10^5 \text{ cm}^{-3}$  with an estimated uncertainty of a factor of  $\pm 3$ . Here, typical errors of factors of  $\pm 2$ ,  $\pm 2$ ,  $\pm 1.5$  and  $\pm 1.5$  were considered for  $k_4$ ,  $\alpha$ ,  $n_+$  and the negative ion abundance ratio  $n_s/n(\text{NO}_3^-(\text{HNO}_3)_2)$ . According to Table 1 the neglect of reactions (7), (11) and (13) may have led to an underestimate of  $n(\text{H}_2\text{SO}_4)$  by a factor of 1.4 at the most.

Continuity equations for ions formed from HSO<sub>4</sub><sup>-</sup>(HNO<sub>3</sub>)<sub>2</sub> and HSO<sub>4</sub><sup>-</sup>H<sub>2</sub>SO<sub>4</sub>HNO<sub>3</sub> can also be used to derive  $n(\text{H}_2\text{SO}_4)$ . However, the rate constants of the switching reactions (8) and (12) have not yet been measured, assuming these to be  $10^{-9} \text{ cm}^3 \text{ s}^{-1}$   $n(\text{H}_2\text{SO}_4)$  values of  $8.3 \times 10^4 \text{ cm}^{-3}$  and  $1.7 \times 10^5 \text{ cm}^{-3}$  are obtained having uncertainties which are again a factor of  $\pm 3$ .

If it is assumed that 50% of the HSO<sub>4</sub><sup>-</sup>H<sub>2</sub>SO<sub>4</sub> ions were formed via reaction (11) rather than via reaction (5) as suggested by the high observed ratio  $n(\text{HSO}_4^-\text{H}_2\text{SO}_4)/n(\text{HSO}_4^-\text{HNO}_3)$ , the former  $n(\text{H}_2\text{SO}_4)$  value of  $8.3 \times 10^4 \text{ cm}^{-3}$  had to be corrected upwards by a factor of about 1.57 which yields  $1.3 \times 10^5 \text{ cm}^{-3}$ . Thus the three derived  $n(\text{H}_2\text{SO}_4)$  values are in excellent agreement within the errors quoted. Therefore a sulphuric acid concentration of  $1.7 \times 10^5 \text{ cm}^{-3}$  with an uncertainty of a factor of  $\pm 3$  is taken to be representative of our data. The corresponding H<sub>2</sub>SO<sub>4</sub> volume mixing ratio is 0.0017 p.p.b.V.

**Fig. 1** Reaction scheme including the major negative ions observed. Neutral reaction partners are indicated besides the arrows. M denotes a collision partner.**Fig. 2** Volume mixing ratios for gas phase sulphuric acid. ●, The measured value. Profile HA 77 is deduced from vapour pressure data given by Hamill *et al.*<sup>12</sup>. Profile H 75 is given by the photochemical diffusive model of Harker<sup>13</sup>. Profile T 79 is given by the photochemical diffusive model of Turco *et al.*<sup>6</sup> which includes aerosol processes.

This experimental value is compared here with predictions of current models for stratospheric sulphur compounds. Major H<sub>2</sub>SO<sub>4</sub> sinks considered are nucleation and photolysis<sup>6</sup>. At heights where the atmospheric temperature is sufficiently low to allow for H<sub>2</sub>SO<sub>4</sub> supersaturation, nucleation should be by far the most efficient sink for gas phase H<sub>2</sub>SO<sub>4</sub>. Then the lifetime of an H<sub>2</sub>SO<sub>4</sub> molecule in the gas phase is determined mainly by the surface area density of the aerosol which, in turn, depends on the concentration and size distribution of the aerosol particles. In these conditions, a lower limit to the sulphuric acid abundance may be estimated from vapour pressure considerations assuming sulphuric acid to nucleate as a binary system (H<sub>2</sub>SO<sub>4</sub>-H<sub>2</sub>O) which leads to the H<sub>2</sub>SO<sub>4</sub> volume mixing ratio profile shown in Fig. 2 (ref. 12). The profile corresponds to a mean temperature profile for mid-latitude spring-autumn conditions as given by the US standard atmosphere (1966) and to a constant water vapour volume mixing ratio of 3.5 p.p.m.V.

An upper limit to the sulphuric acid abundance may be estimated from photochemical-diffusive models neglecting H<sub>2</sub>SO<sub>4</sub>-aerosol processes. As an example, a profile deduced from Harker's model<sup>13</sup> (case 1) is also shown in Fig. 2. When compared with the profile based on vapour pressure considerations, this gives higher values at heights below ~34 km, implying that H<sub>2</sub>SO<sub>4</sub> nucleation in the binary system occurs below this height for the temperature profile used. Considering the lower temperature measured during our balloon experiment at 36.5 km (227 K compared with a value of 239 K as used by Hamill *et al.*<sup>12</sup> the H<sub>2</sub>SO<sub>4</sub> vapour pressure and thus the H<sub>2</sub>SO<sub>4</sub> abundance should have been considerably lower than that shown in Fig. 2. According to the data given in Table 1 of Hamill *et al.*<sup>12</sup>, the difference may be of the order of a factor of 20.

An H<sub>2</sub>SO<sub>4</sub> profile given by a recent comprehensive model which considers photochemistry, vertical transport and aerosol processes of sulphur compounds<sup>6</sup> is also shown in Fig. 2. This profile which is based on the same temperatures as the profile of Hamill *et al.*<sup>12</sup> is similar, as would be expected from a combination of the limiting profiles H 75 and HA 77 (see Fig. 2).

When compared with our experimental sulphuric acid abundance also given in Fig. 2, the model values are much too high. Considering, however, a lower H<sub>2</sub>SO<sub>4</sub> vapour pressure associated with the lower temperature measured during our balloon experiment the expected H<sub>2</sub>SO<sub>4</sub> volume mixing ratio at 36.5 km may have been low as about 0.15 p.p.b.V. which is much closer to the experimental value but still far too high.

Therefore, current models apparently fail to describe the abundance of stratospheric gas phase sulphuric acid. Possible



causes such as an overestimate of the  $\text{H}_2\text{SO}_4$  production or an underestimate of the  $\text{H}_2\text{SO}_4$  loss will now be briefly investigated. The most severe uncertainties of the model computations seem to be associated with (1) poorly known natural fluctuations of the vertical transport of COS strongly affecting the COS abundance and thus the  $\text{H}_2\text{SO}_4$  production around 36 km; (2) unknown photoabsorption coefficients for  $\text{H}_2\text{SO}_4$  which were simply assumed to be the same as those of HCl (ref. 6); (3) considerable uncertainties of  $\text{H}_2\text{SO}_4$  vapour pressure computations<sup>14,15</sup>. Considering a discrepancy between experimental and model  $\text{H}_2\text{SO}_4$  abundances of about a factor of 100 it seems that each of the above uncertainties may be sufficient to account for the discrepancy. Other processes which were not included in the models may be responsible. One is  $\text{H}_2\text{SO}_4$  nucleation in the hypothetical three-component system ( $\text{H}_2\text{SO}_4\text{--HNO}_3\text{--H}_2\text{O}$ ) which is suspected to have a much lower  $\text{H}_2\text{SO}_4$  vapour pressure than the two-component system ( $\text{H}_2\text{SO}_4\text{--H}_2\text{O}$ )<sup>16</sup>. Whether or not  $\text{H}_2\text{SO}_4$  nucleation can account for the low sulphuric acid abundance observed, may be judged from a comparison of  $\text{H}_2\text{SO}_4$  lifetimes in the gas phase. The lifetime for nucleation  $t_N$  is of the order of  $3 \times 10^5$  s at 36.5 km if a typical nuclei concentration of  $10 \text{ cm}^{-3}$  and a typical nuclei size of  $0.04 \mu\text{m}$  (ref. 17) are considered. For comparison, the lifetime  $t$  which can be derived from the experimental sulphuric acid concentration using an  $\text{H}_2\text{SO}_4$  production rate of about  $0.6 \text{ cm}^{-3} \text{ s}^{-1}$  at 36.5 km (deduced from the model of Turco *et al.*<sup>9</sup>) is about  $3 \times 10^5$  s. The agreement of  $t$  and  $t_N$  suggests that at least as far as time constants are concerned the low  $\text{H}_2\text{SO}_4$  abundance observed is consistent with an efficient removal of gas phase  $\text{H}_2\text{SO}_4$  by nucleation. The critical question is whether  $\text{H}_2\text{SO}_4$  was at all supersaturated and thus able to nucleate.

An alternative sink for gas-phase  $\text{H}_2\text{SO}_4$  may be reactions with negative ions followed by the formation of stable ion pairs which ultimately become incorporated into aerosol particles<sup>18</sup>. The  $\text{H}_2\text{SO}_4$  lifetime for this process may be as low as about  $5 \times 10^5$  s at 36.5 km which is close to the inferred  $t$ .

The cause of the discrepancy between experimental and theoretical abundances of gas phase sulphuric acid in the stratosphere can not be identified with certainty. Further *in situ* measurements of  $\text{H}_2\text{SO}_4$  abundances need to be extended in height and they should be carried out in conditions of different stratospheric temperatures. Particularly in summer, when the mean temperature around 36 km may be as high as about 252 K in middle latitudes, the vapour pressure of  $\text{H}_2\text{SO}_4$  should be higher than during our present experiment (227 K) by 2 or 3 orders of magnitude. If such an increase was observed  $\text{H}_2\text{SO}_4$  nucleation could be identified as the cause in question.

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## Stratospheric observation of far IR pure rotational lines of hydroxyl

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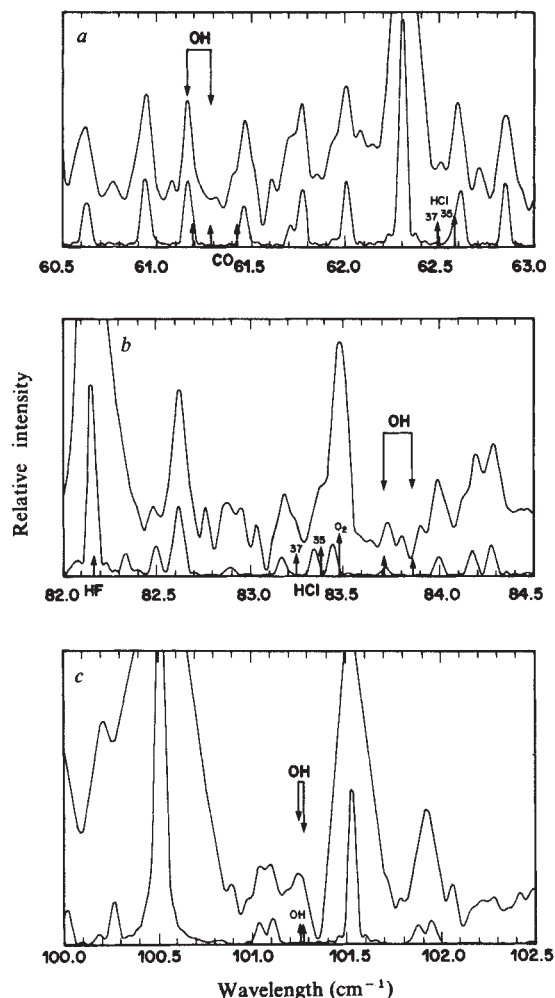
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The hydroxyl radical, OH, has a key role in many atmospheric and ionospheric processes and has been detected in molecular clouds in space. The well-known near IR vibration-rotation bands of this molecule, named after their discoverer, Meinel, were first identified in atmospheric observations<sup>1</sup> while much of the impetus for precise microwave data has come from the identification of radiation in this wavelength range from OH in space<sup>2</sup>. However, although increasingly precise measurements have now been reported from the UV<sup>3</sup> through the IR<sup>4</sup> to the microwave spectral regions<sup>5</sup>, the far IR pure rotational line spectrum has been virtually ignored, and no observations of this relatively simple spectrum have been reported between 5 and  $500 \text{ cm}^{-1}$ . Here we present measurements of spectra between 30 and  $110 \text{ cm}^{-1}$  and deduce stratospheric OH column densities from the observed spectral lines.

We have recalculated<sup>6</sup> the expected pure rotational line positions for all vibrational levels up to and including  $v = 9$  for the ground electronic  $^2\pi$  state and have derived values for the integrated intensity ( $\text{cm}^{-2} \text{ atm}^{-1}$ ) for these lines in the ground electronic and vibrational state at a temperature of 220 K, corresponding to a mean stratospheric temperature. This study showed that the integrated intensity values were very large and suggested that pure rotational lines would be detectable in far IR emission spectra taken in the stratosphere from a balloon platform.

This prediction is important as the amount of hydroxyl in the stratosphere has become an important, even vital, parameter in the current debate on the possible modification of the ozone layer by halocarbons, and measurements of this radical are urgently needed for inclusion in models of ozone behaviour<sup>7</sup>. Only one group has reported direct measurements of OH in the stratosphere<sup>8</sup>, and values obtained by a balloon-borne *in situ* sampling technique, in conjunction with a ground-based resonance fluorescence measurement<sup>9</sup> have shown that the stratospheric OH concentration apparently exhibits large day-time variations. Recent theoretical calculations have shown that while the greatest concentration of OH occurs in the mesosphere at 80 km at night, this peak in the OH density profile moves to the upper stratosphere at 40 km during the day-time<sup>10,11</sup>.

The predicted line strengths for pure rotational lines of OH led to the careful examination of emission spectra of the stratosphere taken during a balloon flight series in 1976 from Yorkton, Saskatchewan, Canada ( $51.3^\circ \text{N}$ ,  $102.3^\circ \text{W}$ ). On one of these flights on 15–16 August, 1979, a simple rapid-scanning Michelson interferometer<sup>12</sup> measured a series of high resolution ( $0.05 \text{ cm}^{-1}$ ) spectra between 30 and  $110 \text{ cm}^{-1}$  of the stratosphere, at an angle of  $84.2^\circ$ , from an altitude of 24.5 km during the evening and sunset periods. An average of 15 of these spectra has been obtained, selected sections of which are presented in the upper parts of Fig. 1a–c. The low altitude and high zenith angle conditions combined to produce very strong  $\text{H}_2\text{O}$  emissions and to enhance weak  $\text{O}_3$  lines. In these spectral regions, the predicted positions for the A-type doublet OH lines are indicated. Figure 1c shows a predicted  $\text{H}_2\text{O}$  and  $\text{O}_3$  spectrum, produced from the AFGL line listing<sup>13</sup>. The present



**Fig. 1** *a-c*, Sections of the stratospheric far IR emission spectrum, observed at a mean altitude of 24.5 km at a zenith angle of 84.2°. These sections are the sum of 15 unapodised spectra computed at a resolution of 0.05 cm<sup>-1</sup>. An equivalent synthetic spectrum for H<sub>2</sub>O and O<sub>3</sub> to a resolution of 0.05 cm<sup>-1</sup> is shown below each section.

spectra are somewhat better than those previously reported from a different flight<sup>6</sup> in having higher resolution and better signal-to-noise ratio in the spectral data. As can be seen from Fig. 1, the OH line positions for the ground electronic and vibrational state are in spectral regions which are relatively free of other lines, and, even though some blending with weak O<sub>3</sub> lines occurs, and other small features in the spectrum are at present unassigned, a tentative identification of pure rotational OH emissions can be made. The lines at 61.29 cm<sup>-1</sup> (with an observed feature at 61.30 cm<sup>-1</sup>), at 83.85 cm<sup>-1</sup> (observed feature at 83.90 cm<sup>-1</sup>) and the combined and strong feature at 101.26 cm<sup>-1</sup> can be used along with the predicted line strengths<sup>6</sup> to yield an average column density of OH of  $8(\pm 3) \times 10^{13}$  molecules cm<sup>-2</sup>. Assumption of a constant mixing ratio above the altitude of 24.5 km yields a value of about 0.1 parts per 10<sup>9</sup> by volume for this mixing ratio, a value which is in reasonable agreement with the values obtained by Burnett<sup>9</sup>.

Thus, even this rather tentative identification is encouraging in indicating that the technique may prove particularly valuable for monitoring this important constituent in the stratosphere, at least during the daytime. Emissions from the higher layers during night-time hours would perhaps make interpretation of the data difficult, although such results might be of interest in their own right for other scientific studies. Higher resolution and sensitivity in the instrumentation during future observations should improve the identification of these pure rotational OH

lines, and angular scanning of the instrument across the atmospheric limb to high zenith angles should allow a profile of OH density to be obtained, a parameter which would be of great value in ozone layer modelling. There is also an urgent need for laboratory measurements to verify the position and integrated intensity values for these pure rotational far IR transitions.

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## Methyl chloride in the stratosphere

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There is much current interest in the chemistry of the stratosphere and the possible impact of chlorine compounds on the Earth's ozone layer. Profiles of anthropogenic compounds such as CFCl<sub>3</sub> and CF<sub>2</sub>Cl<sub>2</sub>, which are emitted from aerosol spray cans, have already been measured in the stratosphere<sup>1</sup>. They confirm that substantial photolysis of these compounds is occurring and thereby releasing chlorine atoms which can catalyse ozone removal. The only natural chlorine containing compound which is likely to enter the stratosphere at significant concentrations is methyl chloride. It is therefore important that its profile be measured and compared with a computed profile to ascertain the likely impact on the ozone layer of naturally occurring chlorine. We report here measurements of the methyl chloride concentration profile up to an altitude of 32 km. The concentration falls off with altitude due to reaction with hydroxyl radicals and it confirms that chlorine of anthropogenic origin is now predominant in the stratosphere.

Lovelock<sup>2</sup> first reported the presence of methyl chloride in the atmosphere at the 1-2 p.p.b. level (1 p.p.b. = 1 part in 10<sup>9</sup> by volume) and showed that its concentrations were highest in air whose origins were oceanic rather than continental. Measurements in seawater confirmed that the ocean could indeed be a significant source in addition to that produced by the smouldering combustion of vegetation. Subsequently studies have provided considerable information on the tropospheric distribution of methyl chloride. Singh *et al.*<sup>3</sup> report mean concentrations of  $611 \pm 84$  p.p.t. (1 p.p.t. = 1 part in 10<sup>12</sup> by volume) and  $615 \pm 103$  p.p.t. in the Northern and Southern Hemispheres, respectively. They find evidence for a large, natural oceanic source and estimate a global source strength of  $3 \times 10^{12}$  g yr<sup>-1</sup>. Rasmussen *et al.*<sup>4-6</sup> find similar values in both hemispheres, with higher values in the atmospheric mixing layer over oceans, strongly indicating an oceanic source. Additionally, they have given



**Table 1** Measurements of methyl chloride in the stratosphere during June 1978 at 44° N

| Probe no. | Altitude | Latitude | MeCl (p.p.t.)     | Comments                           |
|-----------|----------|----------|-------------------|------------------------------------|
| 1         | 32.6 km  | 44° N    | 6.6<br>8.5        | Repeat analysis                    |
| 2         | 31.3 km  | 44° N    | 57.5              |                                    |
| 3         | 28.4 km  | 44° N    | 62.3<br>59.4      | Repeats, also<br>50/52 ratio taken |
| 6         | 19.7 km  | 44° N    | 375               |                                    |
| 7         | 17.5 km  | 44° N    | 476               | 50/52 ratio taken.                 |
|           | 0.1 km   | 51° N    | 590<br>651<br>759 | Harwell ground level<br>samples    |

Methyl chloride levels are given in parts per  $10^{12}$  in air by volume. Measurements at Harwell were made on the day of analysis (20 March 1979).

some attention to Lovelock's suggestion concerning 'slash and burn' agriculture. Elevated concentrations are clearly evident in Kenya and concentrations up to 2 p.p.b. have been reported<sup>7</sup>. In view of the widespread nature of this agricultural practice in tropical latitudes<sup>8</sup>, this source may be of some significance when it is appreciated that much of the transport to the stratosphere takes place through the tropical tropopause<sup>9</sup>. The importance of this source for the stratosphere may not necessarily be reflected in the relative source strengths ( $3 \times 10^{12}$  g yr<sup>-1</sup> for the natural oceanic<sup>2</sup> and  $3-6 \times 10^{11}$  g yr<sup>-1</sup> from the combustion of vegetation)<sup>10</sup>. A measured altitude profile of methyl chloride would certainly help resolve the magnitude of the stratospheric chlorine injection and the relative contributions from the ocean and combustion sources.

To measure methyl chloride in the stratosphere we utilised a balloon probe which collected 10-l samples of air at STP at eight altitudes during the descent phase of the balloon between 32.6 and 17.5 km. The samples were taken over France at a latitude of 44° N. They were cryotrapped into reservoirs contained in the probe using liquid neon and precautions were taken to avoid contamination from the balloon assembly<sup>1</sup>. The air samples in the reservoirs were subsequently transferred into 600 ml stainless steel flasks fitted with metal bellows valves. The pressure in the flasks was typically 4 bar at the time of analysis. Air from the flasks was analysed for a number of components using a gas chromatograph/mass spectrometer combination (Hewlett Packard 5710 GC/VG Micromass 16F MS). Methyl chloride was separated on a 3-m OV 101 column at 20°C and was measured by single ion monitoring at mass 50. The ratio of the signals from mass 50 to mass 52 was measured in two of the stratospheric samples and it compared well with that expected for methyl chloride. About 100 ml of air was used for each analysis and the signal-to-noise ratio was such that 5 p.p.t. could be measured with  $\pm 50\%$  accuracy.

The results for a flight in June 1978 are given in Table 1, and the altitude profile in Fig. 1. Also shown in Table 1 are

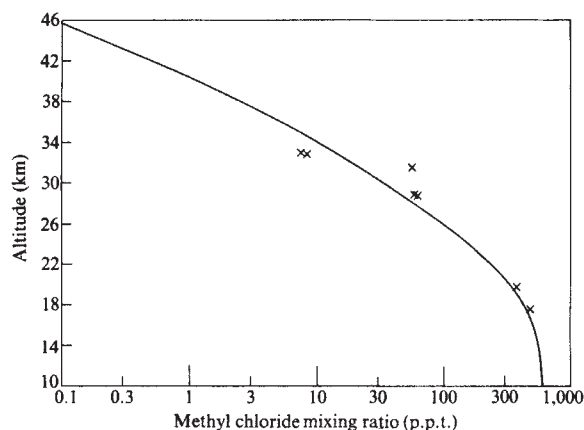
**Table 2** Contributions to the stratospheric chlorine budgets from the various halocarbons

| Halocarbon                        | Chlorine contribution p.p.b. | Comment  | Refs      |
|-----------------------------------|------------------------------|----------|-----------|
| Methyl chloride                   | 0.7                          | Natural  | This work |
| Carbon tetrachloride              | 0.5                          | Man-made | 3, 19     |
| CCl <sub>3</sub> F                | 0.4                          | Man-made | 3, 19, 20 |
| CCl <sub>2</sub> F <sub>2</sub>   | 0.4                          | Man-made | 3, 20     |
| Other organic chlorine compounds* | 0.6                          | Man-made | 21        |
| <b>Total</b>                      | <b>2.6</b>                   |          |           |

\* CH<sub>3</sub>CCl<sub>3</sub>, CHF<sub>2</sub>Cl, C<sub>2</sub>Cl<sub>4</sub>, CHCl<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, C<sub>2</sub>HCl<sub>3</sub>.

measurements made at ground level at Harwell on the day of analyses. These agree well with previous determinations of methyl chloride in the troposphere<sup>3,6</sup>, and confirm that little or no decay had occurred in the containers between flight time and analysis. Unfortunately no data are available between 28 and 20 km because of a malfunction in the balloon probe. In consequence, data are available for only five altitudes, but these show a regular decrease with height in good agreement with our calculated profile. This calculated profile was obtained using a one-dimensional model described elsewhere<sup>11</sup>. This calculation utilised available rate coefficients for the OH + CH<sub>3</sub>Cl reaction<sup>12,13</sup>, UV absorption cross sections for CH<sub>3</sub>Cl (ref. 14) and a lower boundary concentration of 600 p.p.t. at 12 km established on the basis of aircraft measurements<sup>4,5</sup>.

The close agreement between calculation and observation is typical of the behaviour found<sup>11</sup> for other trace gases whose sources are widely distributed throughout the troposphere. This suggests that methyl chloride is predominantly oceanic in origin. A substantial tropical injection from 'slash and burn' agriculture would result in considerable deviation between the observed profile (at 44° N) and the calculated profile (for 30° N) in the lower stratosphere due to polewards transport by the mean



**Fig. 1** Observed and calculated altitude profiles of methyl chloride. X, Measurements at 44° N during June 1978; line, calculated with 1-D model<sup>11</sup> using the  $K_z$  profile of Chang<sup>22</sup>.

circulation and large-scale eddies. In the absence of a significant additional tropical injection, published values of the tropospheric levels of halocarbons can be used to estimate the likely contribution from natural and man-made compounds to the chlorine content of the stratosphere. Table 2 shows that the chlorine from natural methyl chloride is heavily outweighed by chlorine from man-made sources.

Finally it should be noted that the maximum amount of chlorine which can be expected from compounds presently known (2.6 p.p.b.) is substantially lower than the chlorine measured in the form of HCl and ClO in the stratosphere. Recent measurements give values of HCl up to 2 p.p.b. (refs 15, 16) and ClO between 2-8 p.p.b. (refs 17, 18) pointing to an apparent discrepancy which is beyond the scope of the present letter to discuss in detail.

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## Two types of clinoenstatite in Indarch enstatite chondrite

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**Enstatite chondrites are characterised by highly reduced minerals<sup>1,2</sup>. Clinoenstatite and rare olivine are almost Fe-free, carbon occurs as graphite, and Fe, Ni-rich metal contains up to 4% Si. Ca, Ti, Mg and Mn, normally incorporated solely in silicates, are distributed between silicates and sulphides; Ti may also occur as a nitride. We now describe two types of clinoenstatite in the Indarch enstatite chondrite, one luminescing blue and one red. These clinoenstatites have distinct chemical compositions which indicate different redox conditions. The Indarch chondrite is an unannealed mechanical aggregate of diverse materials, and cannot have formed by simple progressive condensation of the solar nebula.**

The Indarch chondrite contains four chondrule types<sup>3</sup>: porphyritic (most abundant), fine-grained, radiating, and barred. Although the minerals of the chondrules appear homogeneous in a petrographic microscope, a cathodoluminescence microscope revealed distinct red and blue types of clinoenstatite crystals. Complex changes of intensity of luminescence occur across some grains, but no chemical variation was found by electron microprobe analysis. Perhaps the intensity change results from crystallographic factors dependent on orientation of twinned and disordered regions. (The polymorphic type of pyroxene was not identified in the polished section but is assumed to be clinoenstatite following ref. 2). Each porphyritic chondrule contains both blue and red crystals but with a strong majority of either one or the other. Radiating chondrules contain large near-euhedral crystals of blue clinoenstatite surrounded by a radiating matrix of red clinoenstatite and other minerals (Fig. 1), perhaps resulting from devitrification. Fine-grained chondrules contain blue clinoenstatite except for a thin rim of red clinoenstatite found in one chondrule. All four types of chondrules are surrounded by a shell of fine-grained material composed of all mineral types found in the meteorite. A shell also occurs around chondrules in some ordinary chondrites<sup>4</sup>.

Electron microprobe analyses (Table 1) were obtained with an ARL-EMX instrument. Mg, Si and Fe were determined with a solid-state detector, 15 kV, 0.1  $\mu$ A beam current, 120 s live time. Synthetic MgSiO<sub>3</sub> glass was the standard for Mg and Si, and P-140 olivine for Fe. Minor elements were determined with spectrometers at 25 kV and 2  $\mu$ A. Counting times for peak and background were chosen to give a precision near 30 p.p.m. (1 $\sigma$ ) for an individual analysis; precision of mean values is lower. Standards were Di<sub>85</sub>Jd<sub>15</sub> glass (Ca, Na, Al), Ca<sub>2</sub>P<sub>2</sub>O<sub>7</sub>(P), Corning glass V (Ti, Cr), Corning glass W(Mn) and Corning glass X(Ni). Secondary fluorescence could contribute but is significant only for Fe analyses made on clinoenstatite lying close

(<0.1 mm) to metal or troilite. Analyses deliberately made on clinoenstatite crystals distant from metal and troilite varied only about 10% in Fe whatever the colour of the clinoenstatite and the type of host chondrule, but random analyses showed some higher values of Fe. The slightly high totals (100.5–101.6) probably result from a small change of thickness of carbon coat between the standard and the unknown; such a small change would have only a trivial effect on atomic ratios.

The minor elements correlate with the luminescence colour but not with the chondrule type. Whereas the red clinoenstatites have higher concentrations of Ti, Al, Cr, Mn and Ca, the blue ones tend to have higher Na. Assignment of valence states to minor elements in pyroxenes depends on crystal-chemical theory, and the following assignments follow currently accepted practice based on ionic potentials.

For blue clinoenstatite, P was assigned to NaMgSiPO<sub>6</sub>, Ti to NaTiAlSiO<sub>6</sub>, Al to NaAlSi<sub>2</sub>O<sub>6</sub> and Cr to NaCr<sup>3+</sup>Si<sub>2</sub>O<sub>6</sub>. The remaining Na could not be accounted for except by assuming a corresponding amount of trivalent iron in the acmite unit NaFe<sup>3+</sup>Si<sub>2</sub>O<sub>6</sub>, or the alternative of vacant sites. Based on 24 oxygen atoms per cell, 0.004–0.017 Fe<sup>3+</sup> atom was needed. The remaining divalent species were coupled with Si. For a perfect analysis, there should be eight Si atoms per cell, apart from the trivial deviation caused by the insignificant amounts of P and Ti, and indeed the observed values of Si lie within experimental error of eight atoms.

For red clinoenstatites, P was assigned to NaMgSiPO<sub>6</sub>, Ti to NaTiAlSiO<sub>6</sub> and the remaining Na or Ti to either NaAlSi<sub>2</sub>O<sub>6</sub> or MgTiAl<sub>2</sub>O<sub>6</sub>. Some Al and Cr was still unused, and it was necessary to use MgAl<sub>2</sub>SiO<sub>6</sub> and MgCr<sup>3+</sup>Si<sub>2</sub>O<sub>6</sub> units. Furthermore, the sum of the remaining divalent ions (assuming all Fe as Fe<sup>2+</sup>) was less than the number of Si<sup>4+</sup> ions. Because the blue clinoenstatites calculated closely to an MTC<sub>3</sub> formula, this discrepancy was not attributed to experimental error, and about one-third of the Fe was converted to the trivalent state. A similar conclusion with slightly different numbers was reached more directly by adjusting the oxygen/metal ratio to the theoretical value of 3:2 by converting some Fe to the trivalent state.

Note that our suggestion of the presence of Fe<sup>3+</sup> is questionable because it depends on crystal-chemical assumptions and analytical error. Direct spectral evidence is needed to confirm or refute the suggestion. A wet chemical analysis for a pyroxene separate from the Kota-Kota enstatite chondrite<sup>5</sup> showed a deficiency of Si, 0.93 wt % FeO, and only 0.03% Fe<sub>2</sub>O<sub>3</sub>. For the present, we note that even if there is no Fe<sup>3+</sup> in the Indarch clinoenstatites, the unequal content of Ti, Cr, and Mn in the red



**Fig. 1** Schematic representation of a fragment of a radiating chondrule. Black areas are red clinoenstatite; hatched regions are blue grains; clear areas are composed of glass or very fine-grained sulphides. The maximum width of the chondrule is 600  $\mu$ m. Thin section from Field Museum collection.



Table 1 Analyses of clinoenstatites

|                                | Indarch |           |         |         |          |         | Kota-Kota |
|--------------------------------|---------|-----------|---------|---------|----------|---------|-----------|
|                                | 1       | blue<br>2 | 3       | 4       | red<br>5 | 6       | 7         |
| P <sub>2</sub> O <sub>5</sub>  | 0.003   | 0.002     | 0.001   | 0.002   | 0.005    | 0.002   | <0.1      |
| SiO <sub>2</sub>               | 59.9    | 59.8      | 59.9    | 60.1    | 60.0     | 59.7    | 58.2      |
| TiO <sub>2</sub>               | 0.002   | 0.002     | 0.005   | 0.059   | 0.052    | 0.027   | 0.03      |
| Al <sub>2</sub> O <sub>3</sub> | 0.051   | 0.057     | 0.038   | 0.267   | 0.293    | 0.147   | 0.32      |
| Cr <sub>2</sub> O <sub>3</sub> | 0.028   | 0.043     | 0.053   | 0.259   | 0.339    | 0.385   | 0.36      |
| FeO <sub>T</sub>               | 0.95    | 0.89      | 0.94    | 0.94    | 0.93     | 0.86    | (0.96)    |
| MnO                            | 0.067   | 0.070     | 0.030   | 0.274   | 0.115    | 0.101   | 0.1       |
| MgO                            | 39.4    | 39.5      | 39.6    | 39.6    | 39.5     | 39.7    | 39.5      |
| NiO                            | 0.005   | 0.003     | 0.004   | 0.011   | 0.003    | 0.006   | 0.034     |
| CaO                            | 0.072   | 0.054     | 0.058   | 0.200   | 0.254    | 0.143   | 0.4       |
| Na <sub>2</sub> O              | 0.109   | 0.068     | 0.095   | 0.041   | 0.062    | 0.005   | 0.05      |
| Total                          | 100.587 | 100.489   | 100.724 | 101.753 | 101.553  | 101.076 | 100.0     |
| cations/24 oxygens             |         |           |         |         |          |         |           |
| P                              | 0.0003  | 0.0002    | 0.0001  | 0.0002  | 0.0006   | 0.0002  | —         |
| Si                             | 7.9977  | 7.9910    | 7.9847  | 7.9463  | 7.9468   | 7.9375  | 7.864     |
| Ti                             | 0.0002  | 0.0002    | 0.0005  | 0.0059  | 0.0052   | 0.0027  | 0.004     |
| Al                             | 0.0080  | 0.0090    | 0.0060  | 0.0416  | 0.0458   | 0.0231  | 0.052     |
| Cr <sup>3+</sup>               | 0.0030  | 0.0045    | 0.0056  | 0.0271  | 0.0355   | 0.0405  | 0.040     |
| Fe <sup>3+</sup>               | 0.0166  | 0.0041    | 0.0130  | 0.0370  | 0.0288   | 0.0568  | —         |
| Fe <sup>2+</sup>               | 0.0893  | 0.0953    | 0.0920  | 0.0670  | 0.0743   | 0.0391  | 0.108     |
| Mn                             | 0.0076  | 0.0079    | 0.0034  | 0.0307  | 0.0129   | 0.0114  | 0.012     |
| Mg                             | 7.8410  | 7.8680    | 7.8680  | 7.8044  | 7.7979   | 7.8666  | 7.948     |
| Ni                             | 0.0005  | 0.0003    | 0.0004  | 0.0012  | 0.0003   | 0.0006  | 0.004     |
| Ca                             | 0.0103  | 0.0077    | 0.0083  | 0.0284  | 0.0361   | 0.0204  | 0.056     |
| Na                             | 0.0277  | 0.0176    | 0.0246  | 0.0105  | 0.0159   | 0.0013  | 0.012     |
| Total                          | 16.0022 | 16.0058   | 16.0066 | 16.0003 | 16.0001  | 16.0002 | 16.100    |

Columns 1–6, Electron microprobe analyses; iron given as FeO. Column 1, Mean of six analyses of blue grains in predominantly red porphyritic chondrule. 2, Mean of six analyses of blue grains in predominantly blue porphyritic chondrule. 3, Mean of five analyses of large blue crystals surrounded by red matrix of radiating chondrule. 4, Mean of four analyses of red grains in predominantly red porphyritic chondrule. 5, Mean of five analyses of red grains in predominantly blue porphyritic chondrule. 6, Mean of six analyses of red matrix of radiating chondrule. 7, Microchemical analyses of bulk pyroxene separate<sup>8</sup>; FeO 0.93, Fe<sub>2</sub>O<sub>3</sub> 0.03 wt%; also K<sub>2</sub>O 0.005.

and blue clinoenstatites must indicate different redox conditions if both clinoenstatites equilibrated with sulphide.

Mechanical aggregation of diverse source materials is required to explain the complex texture and bimodal chemistry of the clinoenstatite. Direct derivation from hypothetical progressive condensation of a gaseous solar nebula can be ruled out, and a complex origin is needed, perhaps involving a rapidly cooled cloud of solid particles, liquid droplets and gas undergoing aerodynamic sorting<sup>6</sup> coupled with settling onto the surface of a growing planetesimal. Perhaps the cloud formed from collision between two planetesimals<sup>7</sup>. Petrographic and mineralogical evidence is accumulating for the presence of assemblages in chemical disequilibrium in all types of unequilibrated chondrites (as reviewed in ref. 7, and emphasised in ref. 8).

Absence of a reaction zone between adjacent blue and red clinoenstatites rules out prolonged annealing of the Indarch enstatite chondrite. If the clinoenstatites really do contain some trivalent iron, they could not be in chemical equilibrium with Si-containing Fe,Ni metal, and mechanical aggregation from two distinct source regions would be implied. Indeed the clinoenstatite-bearing chondrules do not contain large fragments of metal. Tiny grains of metal, too small for routine analysis with an electron microprobe, occur in the porphyritic chondrules, but are tentatively attributed to mechanical aggregation and vapour deposition during coalescence of the red and blue clinoenstatites.

There is undoubted petrographic and chemical evidence of chemical disequilibrium in the Indarch enstatite chondrite. The implied presence of trivalent iron needs testing by spectral techniques, and detailed analyses of trace elements are needed for all minerals. Preliminary study of the Abee, Kota-Kota and Adhi-Kot enstatite chondrites has revealed both red and blue enstatites, and electron microprobe analyses are in progress.

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## Gravity anomalies, block-faulting and Andean-type tectonism in the Eastern Churchill Province

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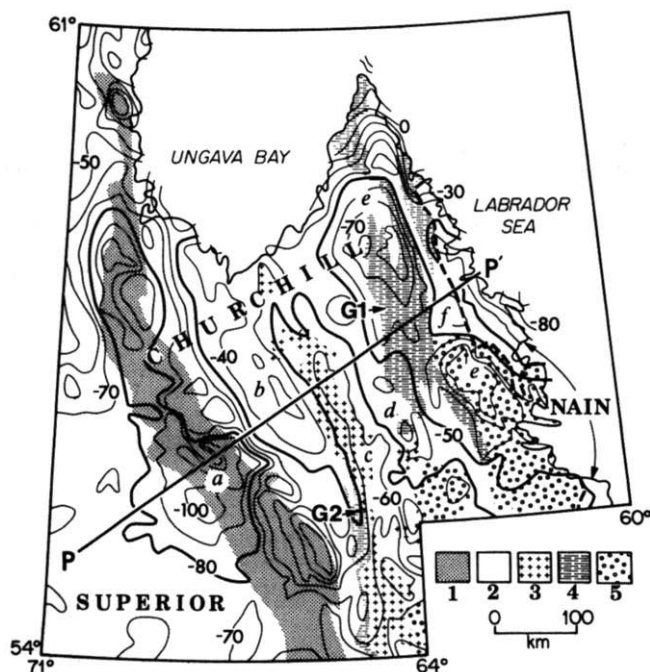
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**Evaluation of the tectonic evolution of Precambrian terrains may be greatly enhanced by selecting appropriate analogues from Phanerozoic examples where the geological history is better understood and where a knowledge of current geodynamic processes is sometimes available. The Churchill Structural Province in the eastern Canadian Shield apparently has a close analogue in the central Andes of Peru where the Nazca plate continues to thrust below the South American plate. We propose that this provides the basis for a tectonic framework that explains several geological features both within the Churchill Province and at the adjacent boundaries with the Superior and Nain provinces.**

The Churchill Structural Province in northeastern Quebec and Labrador is characterised by a distinctive pattern of coextensive, parallel, regional gravity anomalies extending laterally 300–450 km and having widths between 45 and 100 km. These anomalies strike approximately north-northwest and parallel large-scale features, such as the boundaries of the Churchill Province with the Superior Province to the west and the Nain Province to the east, certain major lithological units (Fig. 1), and structural trends apparent from field data and air photographs<sup>1</sup>.

The negative-positive pair of anomalies *a* and *b* (Fig. 1) have been related to structures formed by the collision of Superior and Churchill protocontinents during the Hudsonian Orogeny<sup>2</sup> following subduction of the Superior-bearing plate beneath the Churchill Province. The structures are consistent with a 'basement reactivation' model<sup>3</sup>, in the context of which negative anomaly *a* was explained by downwarped Superior crust and positive anomaly *b* by topography on the Conrad discontinuity, developed during collision, within the thickened Churchill crust<sup>2</sup>. The same dipolar gravity signature is common to several of the structural province boundaries in the Canadian Shield<sup>4</sup>. The type structure of such boundaries, interpreted as sutures, consists of an older crustal block, that thickens towards the suture, in steep contact with a relatively thicker and denser, younger block, both blocks being in approximate isostatic equilibrium.

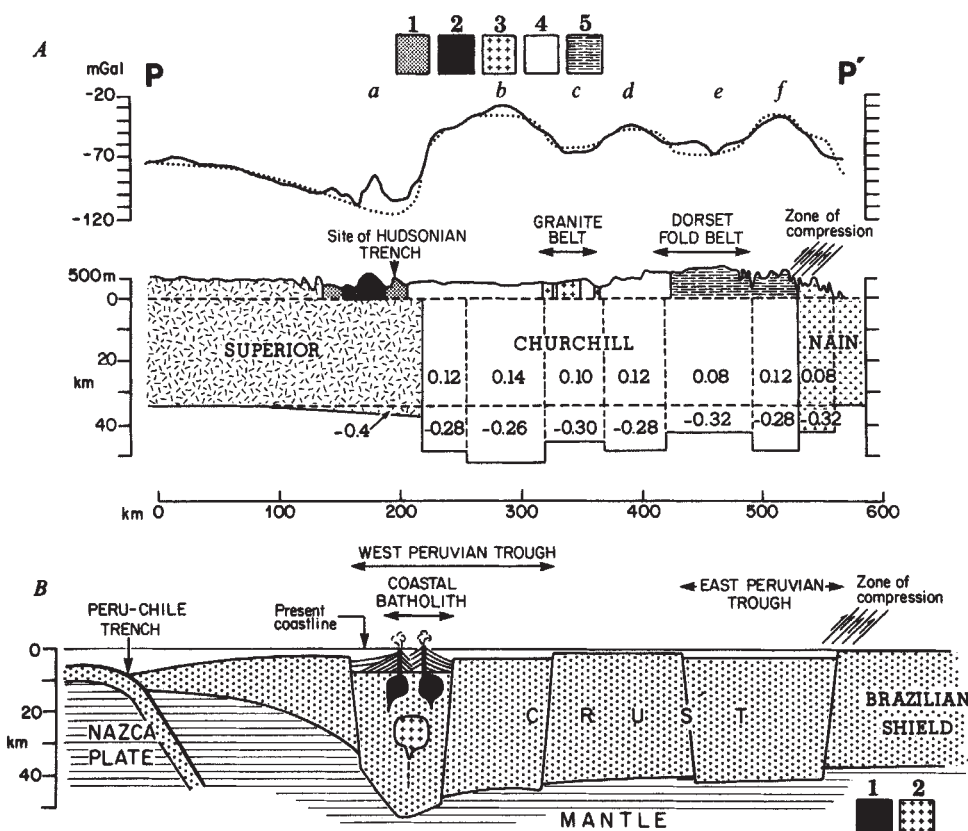
Suggested causes of gravity anomalies east of the boundary pair are topography on the Conrad discontinuity<sup>2,5</sup> and, in some cases, lithological variations<sup>5</sup>. However, there are several lines of evidence, including the extensive nature of the gravity anomalies themselves, to indicate that these regional anomalies are related to major, fault-bounded crustal blocks which are laterally extensive, of differing density and in relative isostatic equilibrium<sup>6</sup> (see model, Fig. 2A); previous interpretations<sup>2,5</sup>



**Fig. 1** Simplified geological map of the northeastern Churchill Province with Bouguer anomalies superimposed. Geological legend for Churchill Province: 1, Labrador Trough; 2, mixed gneisses and migmatites; 3, granites and granodiorites; 4, granulites; 5, anorthosites and adamellites. G1 and G2 indicate areas of granulite. The Churchill-Nain boundary is identified by bold dashed line. Bouguer anomalies are reduced to sea level using a uniform density of  $2.67 \text{ g cm}^{-3}$  and are contoured at an interval of 10 mGal. Bold contours outline anomalies (a-f) discussed in text. P-P' is profile interpreted in Fig. 2A.

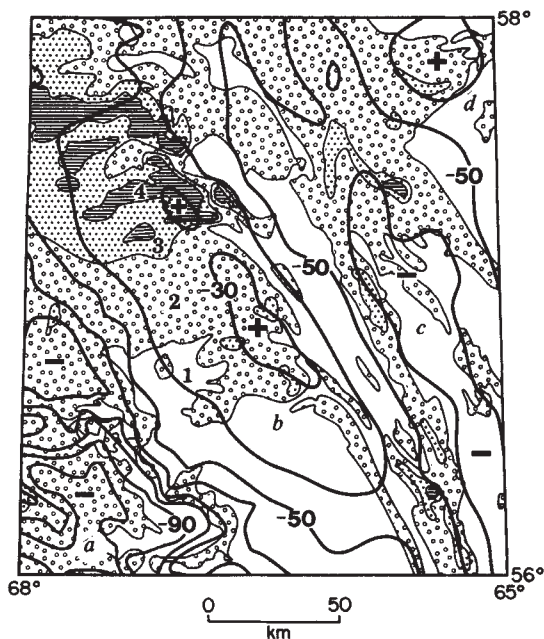
are not necessarily invalidated by this hypothesis but rather become a matter of detail. Geological evidence for all of the large-scale faults interpreted in the model is incomplete, perhaps due to the reconnaissance nature of the mapping and the considerable cover of glacial drift<sup>7,8</sup>. Nevertheless, a 200-km long fault zone has been identified close to the gradient separating anomalies *e* and *f* where it forms much of the eastern boundary of a large area of granulites (G1, Fig. 1). Faults have also been mapped<sup>9,10</sup> along 100 km of the western border of granulites (G2) which correlate with the southern part of positive anomaly *b*. Other prominent faults in the region have north and northwesterly strikes and are indicated by zones of mylonite<sup>7,8</sup>. Relative vertical movements of crustal blocks are indicated by the juxtaposition of high and low grade metamorphic rocks: amphibolite facies terrain to the east of the Labrador Trough underlying anomaly *b* has probably risen 10–15 km relative to lower grade terrain to the west<sup>11</sup>. The eastern limit of this uplifted terrain may coincide with a change in the pattern of aeromagnetic anomalies that occurs roughly along the -40 mGal contour on the east flank of gravity anomaly *b* (Fig. 3) and is considered further evidence in support of a zone of major faulting between blocks underlying anomalies *b* and *c* (Fig. 2A).

The gravity model of the Churchill crust (Fig. 2A) is comparable in morphology and scale to a crustal model of the Andes proposed for central Peru<sup>12</sup> (Fig. 2B). Development of the latter model over the past 100 Myr has been influenced by large-scale vertical faults<sup>12–14</sup> parallel to the Peru–Chile trench that may result from strong coupling between the Nazca and South American plates<sup>15</sup>. Movement along these faults has controlled the following aspects of Andean geology: basin development, batholith emplacement, and the style of deformation of basin fill<sup>12,14</sup>. Basin development and batholith emplacement in the Churchill Province likewise seem to have been controlled by major faults, but any comment regarding deformational styles is precluded by a lack of detailed structural information and the high metamorphic grades. The remains of a former major sedimentary basin, the Dorset Fold Belt<sup>16</sup>, have been identified in the region of negative gravity anomaly *e* (Fig. 1) and continue into Baffin Island. Its Andean analogue is the East Peruvian



**Fig. 2** A, Interpretation of gravity profile P-P' with Churchill Province modelled as several blocks, each in relative isostatic equilibrium. Solid line, observed gravity; dotted line, calculated gravity. Letters a-f identify anomalies discussed in text. Geological legend: 1, metasediments of the Labrador Trough; 2, basic igneous rocks of the Labrador Trough; 3, granitic rocks; 4, mixed gneisses and migmatites; 5, granulites. Density contrasts in  $\text{g cm}^{-3}$ . B, Schematic interpretation of a crustal section across the central Andes at 100 Myr ago (after Myers<sup>14</sup>). Geological legend: 1, basic intrusions; 2, the rising Coastal Batholith.





**Fig. 3** Portion of Churchill Province adjoining the Labrador Trough for which there are published aeromagnetic data. Aeromagnetic anomalies are contoured at 400 nT intervals and increase in intensity from pattern 1 to pattern 4. Superimposed Bouguer anomalies are contoured at 10 mGal interval and outline parts of anomalies *a*, *b*, *c* and *d* (Fig. 1). A change in the crustal character is evidenced in the aeromagnetic anomalies approximately along the line of the -40 mGal Bouguer anomaly contour which lies along the gradient between anomalies *b* and *c*.

Trough (Fig. 2B). Negative gravity anomaly *c* (Fig. 1) coincides with an almost continuous belt of granitic rocks 500-km long. The restriction of granitic rocks to a narrow, linear belt suggests that they have their analogue in the Andean Coastal Batholith which may have been localised by major shear zones and was formed from plutons and sheets intruded into the same tabular belt by repeated cauldron subsidence<sup>12</sup>. Further, the noted absence of syn- and post-orogenic granitic intrusions within the Circum-Ungava belt<sup>17,18</sup> could be explained if the Churchill granite had a similar spatial relationship to the interacting Superior and Churchill plates as the coastal batholith had to the Nazca and South American plates. By this analogy, no granites would be expected within the Labrador Trough, here interpreted as the approximate site of the Hudsonian trench. Rather, granites would be expected some 230 km east of the trough; they are, in fact, centred 70–150 km from the trough (Fig. 1). The smaller distances, most probably, are a result of crustal shortening associated with collision, although the angle of subduction also may have been a contributing factor. Shortening of the Labrador Trough deposits, by thrust faulting and folding, is estimated<sup>19</sup> to have been at least 113 km. The Churchill basement to the east also bears evidence of shortening in the form of tightly pinched synclines, many overturned towards the Superior Province<sup>18</sup>. Radiometric ages (K–Ar) on the Churchill granitic rocks<sup>7</sup> of 1,580 Myr<sup>20</sup> and 1,595 Myr<sup>21</sup> indicate that they were intruded during the late stages of the Hudsonian Orogeny.

According to our model the Churchill–Nain boundary represents the eastern limit of crustal reactivation related to subduction of an oceanic plate, bearing the Superior Province, below an Archean protocontinent comprising what are now the Churchill Province and the North Atlantic Archean Craton<sup>22</sup>. Above the subducted plate, reactivation processes—manifest as the Hudsonian Orogeny—have reset the radiometric clock and transformed Archean crust to Proterozoic crust at the same time imparting to it structural characteristics which distinguish the region as the Churchill Province. The Nain Province, a segment of the now dissected North Atlantic Archean Craton, remained

unaffected. Detailed mapping<sup>23</sup> at the Churchill–Nain boundary has revealed evidence of crustal compression in the form of west dipping thrusts and axial planes of folds attributed to the Hudsonian Orogeny. The structures at this boundary have their counterparts in the Andes where similar compressive tectonics occur in the sub-Andean ranges<sup>24</sup> centred some 500–700 km east of the Peru–Chile Trench. Here, the crust has been compressively deformed from the west against the Brazilian Shield along a belt that marks the eastward limit of intraplate deformation associated with subduction of the Nazca Plate<sup>25,26</sup> (Fig. 2B). There has been recent speculation that the flat geometry of the Nazca Plate, dipping at about 10°, may be responsible for the broad zone of deformation across the central and northern Peruvian Andes<sup>15</sup>. This raises the possibility that the Superior-bearing oceanic plate also subducted at a shallow angle.

Viewed in the light of an Andean analogue several diverse features of the eastern Canadian Shield can be related to a single Proterozoic geodynamic system involving the interaction of two converging plates. Collision tectonics seem to dominate in the region within and bordering the Labrador Trough, but further east it is likely that the geology has evolved in response to the underthrusting of a Superior-bearing oceanic plate.

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## Recrystallisation and electrical behaviour of glacier ice

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Electrical d.c. resistivity measurements made on the George VI Ice Shelf have established the polar nature of Antarctic ice despite saturation by melt-water from surface melt-lakes. Previously it has been suggested that different impurity concentrations are responsible for the extremes in resistivity (a factor of 1,000) between temperate ice (essentially at its pressure melting point) and polar ice (well below its pressure melting point). However, the evidence suggests that impurity levels are similar despite the leaching of contaminants by free water in temperate glaciers, a process which is absent in true polar glaciers. This implies that, although impurities can significantly alter the electrical behaviour of glacier ice, there is a more fundamental mechanism at work. We suggest that this process is one of recrystallisation.

Georesistivity measuring techniques have been applied to the sounding of ice masses many times during the last 25 years. Studies on European glaciers indicate that temperate ice has a resistivity of about  $10^8 \Omega\text{m}$  (refs 1–6). Mono- and polycrystalline ice grown slowly from pure water in the laboratory has given similar values of  $2 \times 10^6$  to  $5 \times 10^8 \Omega\text{m}$  (refs 7, 8). Experiments carried out on ice from polar regions which is well below its pressure melting point have indicated that its resistivity is three orders of magnitude smaller than that of temperate glacier ice, typically between  $5 \times 10^4$  and  $1.5 \times 10^5 \Omega\text{m}$  (refs 9–15), and that the resistivity is temperature and density dependent<sup>10,16</sup>. Electrical experiments undertaken in the laboratory<sup>17,18</sup> on cores taken from both temperate and polar glaciers have confirmed the extreme differences with respect to *in situ* measurements. Recent work has sought an explanation for this marked diversity in resistivity.

Previous *in situ* measurements have been made in the dry-snow and percolation zones of polar ice masses<sup>1–6,12</sup> (as defined by Müller<sup>19</sup>) and in the ablation zone of temperate glaciers<sup>9–15,20</sup>. It was with this background that a comprehensive resistivity survey was carried out on George VI Ice Shelf in the Antarctic Peninsula ( $71^\circ 58' \text{S}$ ,  $67^\circ 34' \text{W}$ ) during January and February 1979. This particular ice shelf undergoes extensive surface melting during the summer and melt-lakes are formed which saturate the firn although the mean annual temperature for the area is  $-11^\circ \text{C}$ <sup>21</sup>. There are two basic layers in the ice shelf. At least the top 25 m of the ice shelf consists of locally accumulated snow which has been modified shortly after its deposition by free water from melt-lakes on the surface. Beneath this layer there is dense dry firn and ice from outlet glaciers flowing from the Palmer Land plateau, a cold ( $-20^\circ \text{C}$ )<sup>22</sup> dry-snow environment.

One of the aims of our study was to determine the extent to which free surface water which is subsequently frozen affects the electrical properties of ice in an area thought to be transitional percolation-soaked zone. We found that resistivities lay in the range  $(5.9 \pm 1.7) \times 10^4 \Omega\text{m}$ , a value which is typical of cold, dry polar ice. This is an important result because it shows that the free water has affected the resistivity only indirectly. In a cold, dry region the transition from fresh snow to dense glacier ice is by a process of densification. The Ross Ice Shelf is an example of such an area and its resistivity profiles reflect a gradual increase in density with depth<sup>13</sup> (mean 10 m density is  $0.425 \text{ Mg m}^{-3}$ ). On the George VI Ice Shelf, where the mean 10-m density is  $0.832 \text{ Mg m}^{-3}$ , our resistivity data imply that the density-depth effect is much less marked than on the Ross Ice Shelf. The water in George VI Ice Shelf melt-lakes freezes in late summer to form extensive thick ice layers of very dense ice ( $0.913 \text{ Mg m}^{-3}$ ) which sandwich bands of firn. The main process of densification is by percolation and refreezing and not simply by compaction. A low frequency resistivity of  $10^5 \Omega\text{m}$  was obtained for ice in a shallow lens formed from melt-water within the last 10 years. This resistivity value is comparable with those of the rest of the ice shelf. Hence, the electrical behaviour of high density ice formed on a cold glacier by compaction is very similar to that of ice formed by the percolation and freezing of melt-water. This implies that the stabilisation of the electrical behaviour of ice is completed within a few years after the ice forms even though recrystallisation continues throughout the life of the ice.

One explanation of the difference in behaviour between samples of temperate and of polar ice (and indeed, between polar ice samples of different depositional age) has been the amount of impurities ( $\text{CO}_2$ <sup>23,24</sup>, and volcanic acids<sup>25</sup>) which remain in the ice after firnification. Yet soluble impurity levels in polar ice and ice in the ablation zone of temperate glaciers are similar and do not differ by orders of magnitude<sup>23,24</sup>. Impurity concentrations, although able to modify electrical behaviour significantly, cannot provide the basic mechanism which accounts for the difference between temperate and polar ice<sup>26</sup>.

We wish to draw attention to the consistency of polar ice resistivities even where melt-water is present. Two potentially important mechanisms which operate in temperate glaciers and

which are reduced or absent in polar ice masses are the complete removal of melt-water runoff and the extent of recrystallisation that occurs in response to stress. We now suggest that these factors may be responsible for the differences in the electrical behaviour between polar and temperate ice.

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## Prediction and test of the effects of interspecific competition

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Although interspecific competition is of major importance in ecological and evolutionary theory<sup>1</sup>, there has been no critical study of whether indirect estimates of niche overlap accurately reflect the effects of competition on fitness as indicated by reproductive output. I report here that competition from jackdaws, *Corvus monedula*, significantly reduced reproductive output of magpies, *Pica pica*, due to starvation of nestlings and a high rate of nest predation. The competition coefficient as estimated from these direct effects ( $\alpha = 0.43$ ) was only slightly smaller than predicted by niche overlap values based on morphological similarity, habitat distribution and feeding technique ( $\alpha = 0.58$ ), or food composition ( $\alpha = 0.54$ ).

The study was carried out in a south Swedish grassland habitat with scattered young pine plantations, most of them containing one pair of magpies. Niche overlap was estimated in two ways. First, the percentage overlap<sup>2</sup> in the frequency distribution of jackdaws and magpies foraging in different habitats (areas with heath, grassland, tall herbs and pines), and using different feeding techniques (gleaning, probing and chasing) was determined. The ratio of bill sizes in the two species was also calculated. The product of all these values gave an estimate of overall niche overlap of  $\alpha = 0.58$ . Second, samples of food brought by the parents to their nestlings were identified to species level, except for cereals and a few unimportant categories. The percentage overlap in frequency distribution of the food of the two species yielded  $\alpha = 0.54$ .

If jackdaws and magpies compete for limited resources, one would expect the effects of competition on reproductive output to be closely related to these overlap values, provided the values



**Table 1** Breeding results of experimental and control pairs of magpies

|                                                           | Experimental group | Control group | P (two-tailed) |
|-----------------------------------------------------------|--------------------|---------------|----------------|
| Start of laying (median date)<br>day 1 = 1 April          | 20                 | 21            | NS             |
| Clutch size                                               | 6.50 ± 0.30        | 6.48 ± 0.18   | NS             |
| Mean weight of clutch                                     | 64.7 ± 4.0         | 67.1 ± 2.3    | NS             |
| Mean of mean nestling weight<br>within brood, age 21–25 d | 182 ± 7            | 208 ± 4       | < 0.02*        |
| No. of started breedings (N)                              | 18                 | 38            |                |
| No. of hatched<br>clutches (% of N)                       | 10<br>(56%)        | 23<br>(61%)   | NS             |
| No. of broods<br>with ≥ 1 fledged<br>young (% of N)       | 3<br>(17%)         | 16<br>(42%)   | < 0.04†        |
| No. of fledged young per<br>successful brood              | 2.0 ± 0.58         | 4.0 ± 0.26    | < 0.05*        |
| No. of fledged young per<br>breeding attempt              | 0.33 ± 0.20        | 1.68 ± 0.34   | < 0.001        |

Figures are given ± s.e. NS, not significant.

\* Mann-Whitney U test.

† Fischer exact probability test.

give an accurate measure of niche overlap. The values are directly comparable to the competition coefficient because the two species have almost identical weights and, therefore, also identical consumption rates.

This prediction was tested with a 2-yr experiment. Three jackdaw nest-boxes were put up in each of a number of experimental magpie territories. The nest-boxes were occupied immediately by jackdaws. Pairs of magpies served as controls and experimental birds in alternate seasons, so that their breeding success in the two situations could be compared. Combined data from all experimental and control pairs for the two seasons are shown in Table 1, and the reproductive output of individuals in experimental and control conditions is shown in Table 2: this comparison can be made only for birds that were individually marked and survived both breeding seasons. The start of laying and the size and weight of clutch were similar for the two groups, whereas experimental pairs produced fewer young, with lower weights at fledging, than did control pairs.

The competition coefficient for the effect of jackdaws on magpie reproduction ( $\alpha_{mj}$ ) is defined by:

$$N_m = C - \alpha_{mj} \times N_j - D$$

where  $N_m$  and  $N_j$  are equilibrium numbers (adults plus nestlings) of magpies and jackdaws respectively,  $C$  is carrying capacity and  $D$  is the impact on magpie reproduction of competitors other than the jackdaw. Assuming identical  $C$  and  $D$  for experimental and control territories, the competition coefficient may be determined from

$$\alpha_{mj} = \frac{N_m(\text{control}) - N_m(\text{exp.})}{N_j(\text{exp.}) - N_j(\text{control})}$$

This coefficient can be calculated on the following assumptions. (1) The difference in the numerator amounts to 1.35 magpie nestlings (Table 1). As Fig. 1 shows, the difference in reproductive output between pairs of experimental and control magpies is established well before the end of the nestling period. Thus, equilibrium seems to be attained. (2) The time spent by foraging adult magpies and jackdaws in 25 × 25-m plots was recorded 26–30 d after hatching, during 7 h of observation in each of three experimental and two control territories. Magpie foraging areas (≥ 1% of total magpie foraging time) were of similar size in experimental and control pairs, on the average 0.96 and 1.00 ha, respectively. The total number of 'jackdaw feeding minutes' in the magpie foraging areas of experimental pairs were 411, 563 and 575 and of control pairs 24 and 50. This corresponds to an average number of 1.23 foraging jackdaws in experimental magpie territories and 0.09 in control ones. As

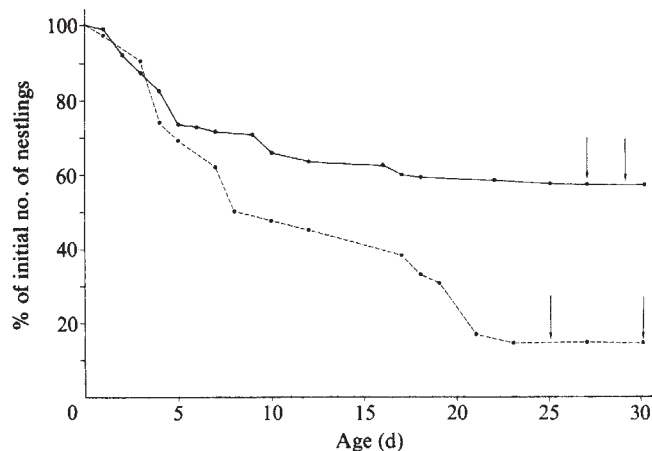
magpies foraged during 91% of total observation time, these figures are corrected to 1.35 and 0.10, respectively. Consequently, there was a mean difference of 1.25 foraging adult jackdaws between experimental and control magpie territories. (3) The mean number of nestlings in the jackdaw nest-boxes was 2.29 (s.e. = 0.19). Thus, 1.25 adult jackdaws provided food, both for themselves and for, on average,  $1.25 \times 0.5 \times 2.29 = 1.43$  nestlings. (4) As magpies and jackdaws hatch almost simultaneously and their nestlings weigh approximately the same, the energy requirements of the nestlings of the two species are almost identical. Thus, for the denominator in the above quotient to be directly comparable to the numerator (= 1.35 magpie nestlings) the denominator should be transformed into number of jackdaw nestlings. It is estimated that energy consumption of an adult jackdaw exceeds that of a nestling by about 35 to 40% (refs 3–4). Thus the difference between the numbers of jackdaws at experimental and control magpie territories, 1.25 and 1.43 nestlings, will be equivalent to a total of 3.15 jackdaw nestlings. As a result

$$\alpha_{mj} = \frac{1.35}{3.15} = 0.43$$

This competition coefficient agrees fairly well with the predictions of niche overlap, based on circumstantial evidence.

A directional index<sup>5</sup> [ $\alpha_{mj} = (\sum_h p_{jh} p_{mh}) / (\sum_h p_{mh}^2)^{-1}$ ] has often been used to estimate competition coefficients. Using this index and assuming equal extraction rates for all kinds of prey and equal consumption rates for the two competitors<sup>6</sup>, values of 1.04 (based on bill dimensions, habitat distributions and feeding techniques) and 0.69 (based on food composition) are obtained for the competitive effect of jackdaw on magpie, and 0.59 and 0.71, respectively, for the reverse effect. This method leads to high expected competition coefficients, and it may not be so useful in natural conditions<sup>7</sup>, as also indicated by the results reported here.

The competitive effects of jackdaws foraging in magpie territories were evident in the following sequence (Tables 1 and 2 and Fig. 1). The presence of jackdaws had no effect during the earlier stages of magpie breeding, before hatching. This is probably explained by a slight difference in time of laying, the magpie being 5 d ahead of the jackdaw, and the relatively small energy demands of the birds during the early phase of breeding. There was no sign of interspecific aggression during this period. After hatching, experimental magpies lost nestlings to a markedly higher degree than did control pairs. Both starvation and nest predation (chiefly by hooded crows, *Corvus cornix*) caused these losses, and the effects of these two factors were of similar magnitude. During this period magpies became highly aggressive—101 attacks on foraging jackdaws were recorded



**Fig. 1** Mortality of experimental (---, see text) and control (—) magpie broods. Arrows delimit periods of observation on jackdaw distribution in magpie feeding areas. In the control group there were 115 hatched young, and in the experimental group, 42.

**Table 2** Breeding results of individual females in experimental and control conditions, in the same territory

| Female | Start of laying (day 1 = 1 April) |    | Clutch size |   | Weight of clutch |      | No. of fledged young       |   |
|--------|-----------------------------------|----|-------------|---|------------------|------|----------------------------|---|
|        | E                                 | C  | E           | C | E                | C    | E                          | C |
| 1      | 17                                | 8  | 6           | 7 | 45.0             | 50.4 | 0                          | 3 |
| 2      | 20                                | 23 | 6           | 6 | 60.0             | 61.2 | 2                          | 3 |
| 3      | 25                                | 29 | 5           | 6 | 52.5             | 63.0 | 0                          | 3 |
| 4      | 26                                | 28 | 7           | 7 | 68.6             | 70.0 | 1                          | 1 |
| 5      | 13                                | 11 | 7           | 7 | 77.0             | 79.1 | 0                          | 0 |
| 6      | 21                                | 16 | 6           | 6 | 64.2             | 62.4 | 0                          | 0 |
| 7      | 20                                | 28 | 8           | 7 | —                | —    | 0                          | 4 |
| 8      | —                                 | —  | —           | — | —                | —    | 0                          | 0 |
| 9      | —                                 | —  | 5           | 6 | —                | —    | 0                          | 4 |
| P      | NS                                |    | NS          |   | NS               |      | $t_8 = 2.78$<br>$P < 0.05$ |   |

—, Data missing; NS, not significant.

during 35 h of observation. The jackdaws probably depleted available food in the magpie territories, thereby causing starvation of the magpie nestlings. Food depletion may have forced the parent magpies to forage farther from their nest than normal, thereby reducing the possibilities of defending their nest against predators. Furthermore, hungry nestlings beg for food loudly, thus increasing the risk of predator attraction.

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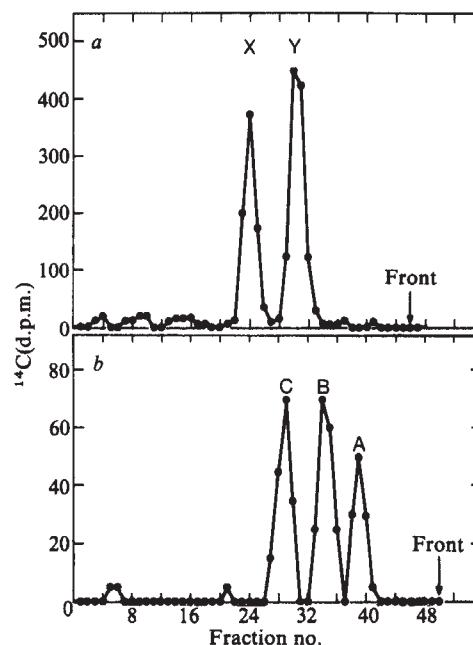
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## Plants metabolise ethylene to ethylene glycol

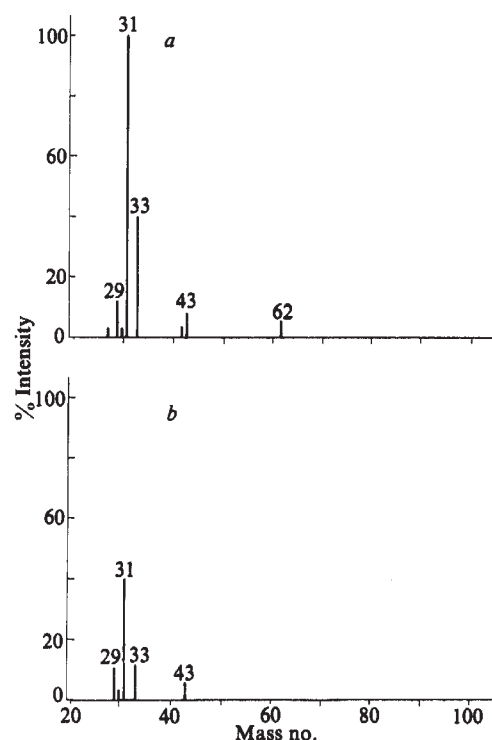
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Ethylene is a natural plant growth regulator often produced in sufficient quantities to alter cellular and developmental processes in a characteristic hormonal manner<sup>1</sup>. Almost all phases of plant development are affected, including germination, growth, flowering, dormancy, abscission, senescence and sex expression. The earlier view that ethylene is metabolically inert<sup>2–4</sup> has been shown to be untenable<sup>5–7</sup>; low but readily detectable rates of ethylene metabolism have been observed in several plant tissues. These and other studies<sup>8–10</sup> indicate that this metabolism does not represent a system for the removal of the ethylene but instead represents the initial biochemical events in the action of ethylene in the plant. Here we report that one of the stable end-products of this ethylene metabolism is ethylene glycol, while for the ethylene mimic propylene, it is 1,2-propanediol.



**Fig. 1** Separation of neutral metabolites of *a*, <sup>14</sup>C-ethylene and *b*, <sup>14</sup>C-propylene by paper chromatography with butanol:acetic acid:water 3:3:2.



**Fig. 2** Identification of ethylene metabolite Y (*b*) by mass spectroscopy. Mass spectra of metabolite isolated from ethylene-exposed pea tissue and of authentic ethylene glycol (*a*) were obtained by scanning effluent peaks from a Chromosorb 101 GC column, heated from 75 °C to 190 °C at 15 °C min<sup>-1</sup>, with a Vacuum Generators 16F mass spectrometer. Because of the low concentration of metabolite in the extract its three weakest mass peaks were below the detection threshold and hence were absent from its spectrum.

Pea seedlings were surface sterilised as described previously<sup>5</sup> and germinated in the dark in sterile vermiculite. After 5 d the upper halves of the seedlings were removed, placed in glass stoppered flasks with 4 ml distilled water and exposed in the dark at 24 °C to 10 μl l<sup>-1</sup> <sup>14</sup>C-labelled ethylene (Amersham,



120 mCi mmol<sup>-1</sup>) purified by preparative gas chromatography<sup>11</sup>. Incubation flasks contained a side-well with 1 ml 1 M NaOH to trap evolved CO<sub>2</sub>. After exposure for 1 or 2 d, epicotyls were removed and a 50% ethanol extract of the tissue was prepared. After centrifugation the extract was freed of acids and bases by passage through columns of cationic and anionic exchange resins (AG50W-X8 and AG1-X8, Bio-Rad). Paper chromatography of the resulting neutral extract revealed two <sup>14</sup>C-labelled metabolites (Fig. 1a). The faster moving metabolite Y was investigated first because short-term labelling and pulse chase experiments indicated that it was formed before X. When compared to various neutral standard compounds, including oxidation products of ethylene, it always co-migrated with ethylene glycol (Table 1).

For confirmation by mass spectrometry, seedlings were grown in large trays for 5 d and exposed to 1,000 µl l<sup>-1</sup> unlabelled ethylene for 2 d. Epicotyls were then collected and from 2,700 g fresh weight of tissue a neutral ethanol extract was prepared, spiked with <sup>14</sup>C-labelled metabolite Y, and purified for gas chromatography-mass spectrometry (GC-MS) using ion-exchange chromatography, acetonitrile precipitation and HPLC (silica column, mobile phase of butyl chloride: methanol: H<sub>2</sub>O, 830:170:5). The mass spectrum of the Y peak emerging from a Chromosorb 101 GC column was of low intensity but essentially identical to that of authentic ethylene glycol (Fig. 2).

In similar studies with the ethylene mimic propylene, exposure of pea epicotyls to 10 µl l<sup>-1</sup> of purified <sup>14</sup>C-labelled propylene (Amersham, 3 mCi mmol<sup>-1</sup>) formed three <sup>14</sup>C-labelled metabolites (Fig. 1b). Co-chromatography with authentic material clearly showed that propylene metabolite A was 1,2-propanediol (Table 2), and GC-MS analysis of a purified sample from pea epicotyls provided positive identification (Fig. 3).

Ethylene metabolite X and propylene metabolite C have been identified as glucose conjugates of ethylene glycol and 1,2-propanediol, respectively, and treatment of either with β-glucosidase yields the corresponding glycol. The formation of metabolites X and Y appears to be a general phenomenon

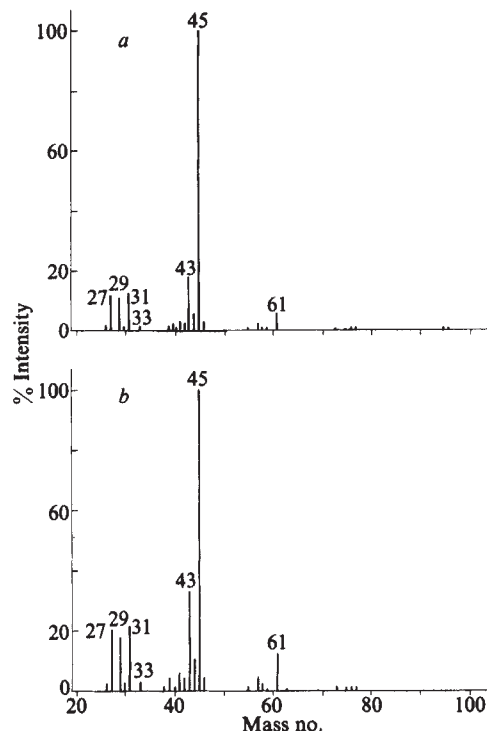


Fig. 3 Identification of propylene metabolite A (b) by mass spectroscopy. Mass spectra of purified metabolite from propylene-exposed pea tissue and of authentic 1,2-propanediol (a) were obtained as in Fig. 2.

associated with the physiological chemistry of ethylene in plants as we have detected them in a variety of tissues exposed to <sup>14</sup>C<sub>2</sub>H<sub>4</sub>, including pea epicotyl tissue, carnation ovary tissue, morning glory flowers, cotton abscission zone segments and tomato fruit.

Table 1 Co-chromatography of ethylene metabolite Y and ethylene glycol

| Chromatographic system | Solid phase                   | Mobile phase                                              | Retention time or <i>R<sub>f</sub></i> value |                               |
|------------------------|-------------------------------|-----------------------------------------------------------|----------------------------------------------|-------------------------------|
|                        |                               |                                                           | <sup>14</sup> C-ethylene metabolite Y        | Ethylene glycol               |
| Liquid                 | Silica column                 | Acetonitrile: water (85:15)                               | 6.50 min                                     | 6.41 min                      |
| Liquid                 | Silica column                 | Butyl chloride: methanol: water (75:25:0.5)               | 9.90 min                                     | 9.82 min                      |
| Liquid                 | Amine-bonded silica column    | Acetonitrile: water (199:1)                               | 3.70 min                                     | 3.70 min                      |
| Gas                    | Chromosorb 101 column, 200 °C | Helium                                                    | 2.45 min                                     | 2.46 min                      |
| Thin layer             | Cellulose                     | Ethyl acetate: acetic acid: formic acid: water (18:3:1:4) | 0.72 ( <i>R<sub>f</sub></i> )                | 0.72 ( <i>R<sub>f</sub></i> ) |
| Paper                  | Cellulose                     | Butanol: acetic acid: water (3:3:2)                       | 0.64 ( <i>R<sub>f</sub></i> )                | 0.65 ( <i>R<sub>f</sub></i> ) |

<sup>14</sup>C-ethylene metabolite Y was detected by scintillation counting in all systems. Ethylene glycol was detected by monitoring the refractive index in liquid systems, by flame ionisation in the gas system, and by scintillation counting of authentic <sup>14</sup>C-ethylene glycol in thin layer and paper systems.

Table 2 Co-chromatography of propylene metabolite A and 1,2-propanediol

| Chromatographic system | Solid phase                   | Mobile phase                                | Retention time or <i>R<sub>f</sub></i> value |                               |
|------------------------|-------------------------------|---------------------------------------------|----------------------------------------------|-------------------------------|
|                        |                               |                                             | <sup>14</sup> C-propylene metabolite A       | 1,2-Propanediol               |
| Liquid                 | Silica column                 | Butyl chloride: methanol: water (75:25:0.5) | 7.59 min                                     | 7.65 min                      |
| Liquid                 | Silica column                 | Acetonitrile: water (98:2)                  | 8.08 min                                     | 8.00 min                      |
| Liquid                 | Nitrile-bonded silica column  | Butyl chloride: methanol: water (85:15:0.5) | 7.87 min                                     | 7.85 min                      |
| Gas                    | Chromosorb 101 column, 200 °C | Helium                                      | 2.90 min                                     | 2.90 min                      |
| Thin layer             | Silica                        | Butyl chloride: methanol: water (75:25:0.5) | 0.50 ( <i>R<sub>f</sub></i> )                | 0.48 ( <i>R<sub>f</sub></i> ) |
| Thin layer             | Silica                        | Acetonitrile: water (95:5)                  | 0.58 ( <i>R<sub>f</sub></i> )                | 0.60 ( <i>R<sub>f</sub></i> ) |

<sup>14</sup>C-propylene metabolite A was detected by scintillation counting in all systems. 1,2-Propanediol was detected by monitoring the refractive index in liquid systems, by flame ionisation in the gas system, and by I<sub>2</sub> vapour staining in thin layer systems.

To our knowledge this is the first report demonstrating that higher plants oxidise ethylene to ethylene glycol. The importance of this oxidative process stems from its apparent direct involvement in the mechanism of action of this hormone<sup>7</sup>. It has been suggested<sup>6</sup> that this metabolism might occur at a Cu-containing receptor site, and this notion is supported by the sensitivity of this system to Cu chelators, COS and CS<sub>2</sub> (ref. 8). In nonbiological aqueous systems Cu-C<sub>2</sub>H<sub>4</sub> complexes can undergo oxidation to yield ethylene oxide<sup>12</sup>, and in *Vicia faba* L. cotyledons<sup>13</sup> ethylene was recently shown to be rapidly converted to this product. Although this rapid conversion seems to be unique to this tissue (ref. 14 and M. A. Hall, personal communication), it is of interest because ethylene oxide is a likely precursor of ethylene glycol. In conditions like those found in *Vicia faba* cotyledons, where ethylene is rapidly metabolised, perhaps ethylene oxide escapes before undergoing hydrolysis, or this latter step may be blocked in this tissue. Precedent for this can be found in microbial systems where epoxidation of ethylene apparently occurs but not glycol formation<sup>15</sup>.

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## Light-dependent synthesis of cholecalciferol in a green plant

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Cholecalciferol (vitamin D<sub>3</sub>) and several of its metabolites participate in the regulation of calcium and phosphorus metabolism in higher vertebrates (see refs 1, 2 for reviews). In contrast to the synthesis of ergocalciferol, the vitamin D of plant origin, cholecalciferol synthesis has been considered to be confined to skin lipids of vertebrates. Its 6,7-*cis*-intermediate, precalciferol<sup>3</sup>, is formed by a photoreaction (wavelength 250-310 nm) from 7-dehydrocholesterol in or on skin tissue. Recently, however, a metabolite of cholecalciferol, 1,25-dihydrocholecalciferol, previously known to be synthesised only in mammalian and avian kidneys, has been detected in at least two species of Solanaceae<sup>4,5</sup>. Furthermore, Rambeck *et al.*<sup>6</sup> showed that cholecalciferol occurs in the common grass, *Trisetum flavescens*, which had been identified by Dirksen *et al.*<sup>7</sup> as the cause of callosities in grazing cattle. We now demonstrate that cholecalciferol is formed in *T. flavescens* only in the presence of UV light, thus suggesting a similar path of synthesis in plants to that which occurs in vertebrates.

For the present studies, *T. flavescens* and other grasses were sown in a dark room in wooden frames containing finely sieved soil and covered by cubic tents of aluminium foil to exclude all radiation from the outside. Inside, Grolux lamps of 30 W each were placed 60 cm above the soil surface. Spectral energy distribution as measured at soil level was: 780-400 nm, 97.5%; 400-315 nm, 2.1%; 315-280 nm, 0.4%. To exclude UV light entirely, yellow Plexiglass filters, which absorb wavelengths <490 nm, could be inserted horizontally beneath the lamps. Growth, as judged by appearance and dry matter yield per m<sup>2</sup>, was not noticeably influenced by the absence of UV light. The grass was cut after 4-6 weeks and freeze dried taking care to prevent exposure to any source of light.

**Table 1** Influence of *T. flavescens* grown with and without UV light on rat inorganic serum phosphorus (mg P per 100 ml)

|                                                               | Test 1    | Test 2    | Test 3    |
|---------------------------------------------------------------|-----------|-----------|-----------|
| Concentration of <i>T. flavescens</i> dry matter in test diet | 6%        | 3%        | 2%        |
| + UV grown                                                    | 4.2 ± 0.9 | 4.5 ± 0.6 | 3.1 ± 0.5 |
| - UV grown                                                    | 2.6 ± 0.5 | 2.7 ± 0.4 | 2.2 ± 0.6 |
| Negative control                                              | 1.4 ± 0.1 | 2.5 ± 1.1 | 1.7 ± 0.4 |
| Negative control + vitamin D <sub>3</sub>                     | 3.3 ± 0.7 | 3.7 ± 0.3 | —         |

Weanling rats are placed on a vitamin D-free diet containing ~0.5% total P. Calcium concentration is ~0.4% for 14 days and is reduced to ~0.1% during the third week. The experimental diets, containing the respective vitamin D sources and ~4% Ca, are then given for 3 days. This change in Ca intake leads to a sudden drop in serum P from 9-12 to 1.5-2.5 mg per 100 ml. Orally administered vitamin D reduces this drop in P<sub>i</sub> serum levels. P<sub>i</sub> was measured 65 h after the diet switch. Six individually kept rats were used for each treatment.

The vitamin D activity of the samples was tested by the same procedure previously used for the purification and isolation of cholecalciferol from *T. flavescens*<sup>6</sup>. This test uses an acute, drastic drop in inorganic serum phosphorus level which is induced by dietary means in the rat and can be prevented by administration of vitamin D (see Table 1 legend). *T. flavescens* grown without UV light showed significantly less, but still some vitamin D activity compared with *T. flavescens* grown without the filters in three independent replications (Table 1). As we suspected that traces of light may have led to some vitamin D activity, in the following experiments, the final steps of preparation were carried out in total darkness. As an additional control, an equivalent amount of diethyl ether extract from *T. flavescens* grown without UV was tested after a short exposure to a UV lamp.

The results, shown in Table 2, indicate that vitamin D activity is completely dependent on UV exposure, and, in addition, that a single exposure of quite short duration, as was the case with the extract, results in considerable vitamin D activity. Therefore, UV radiation is unlikely to be a limiting factor of cholecalciferol synthesis in this plant even in natural growing conditions.

Further experiments, to be reported in detail elsewhere, showed that the vitamin D activity is mainly confined to the leaves<sup>8</sup>. The concentration of cholecalciferol as estimated by the quail eggshell test<sup>9</sup>, rat test, GC and HPLC ranged from about 0.05 to 0.15 mg per kg of whole plant dry matter. Cholecalciferol concentration seems to be independent of the stage of vegetation, first or second cutting, soil, geographical area and altitude above sea level. Differences are due to the proportions of young leafy parts. Five different breeds of *T. flavescens* were of similar activity. However, samples of *Trisetum elatior* (*Avena elatior*, *Arrhenatherum elatius* L.), closely related to *T. flavescens*, were entirely inactive. A very small, insignificant elevation of inorganic serum phosphorus was obtained in our tests from



**Table 2** Influence of UV light on vitamin D activity of *T. flavescens* and *T. flavescens* extract as measured by inorganic serum phosphorus (mg P per 100 ml)

|                                                                                             | Test 1    | Test 2    |
|---------------------------------------------------------------------------------------------|-----------|-----------|
| Concentration of<br><i>T. flavescens</i> dry matter<br>(or extract aliquot)<br>in test diet | 3%        | 3%        |
| + UV grown                                                                                  | 3.7 ± 0.6 | —         |
| — UV grown                                                                                  | 2.0 ± 0.3 | —         |
| — UV grown extract                                                                          | —         | 4.0 ± 0.5 |
| UV exposed                                                                                  |           |           |
| Negative control                                                                            | 2.4 ± 0.6 | 2.6 ± 0.6 |
| Negative control + vitamin D <sub>3</sub>                                                   | 4.3 ± 0.7 | 3.5 ± 0.6 |

For methods see Table 1 legend. The extract from lyophilised *T. flavescens* (grown without UV) was prepared by diethyl ether extraction as described by Rambeck *et al.* and placed for 3 min under a laboratory UV lamp<sup>6</sup>.

samples of *Dactylis glomerata*. This widely occurring grass had been reported earlier<sup>10</sup> to contain cholecalciferol, although the concentration was not mentioned. Cholecalciferol or its immediate precursors had not been detected in a study of sterol distribution in pasture grasses, which included *T. flavescens*<sup>11</sup>. This may have been due to the low concentration, which necessitates rather refined techniques for its detection<sup>6</sup>.

Our results are the first demonstration of a UV light-dependent synthesis of cholecalciferol in an autotrophic plant. (We have previously shown<sup>6</sup> that the total vitamin D activity of *T. flavescens* can be attributed to cholecalciferol rather than to any of its metabolites.) Our findings may have some relevance to hitherto unresolved questions concerning the ultimate origin of cholecalciferol in the livers of marine fish. Holick *et al.*<sup>12</sup> recently reported that some marine fish are incapable of synthesising cholecalciferol in the absence of UV light and may not even metabolise cholecalciferol. Thus, cholecalciferol may be a rather inert constituent of the marine food chain, produced by as yet unidentified species of the autotrophic phytoplankton in the upper strata of the oceans, where some penetrating UV radiation may suffice for its synthesis in plant cells. The functions of 'vitamin D' in plant cells are unknown, but a stimulatory effect of high concentrations of vitamin D analogues, including cholecalciferol, on rhizogenesis was recently reported by Buchala and Schmid<sup>13</sup>.

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## A square bacterium

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I have come across a bacterium which has the form of a thin square sheet. In most bacteria such a shape would be precluded by the osmotically-generated internal hydrostatic pressure but this organism, found in a saturated brine pool, has little or no cell turgor pressure. Its shape is probably determined by the pattern in which the cell envelope particles assemble. These square bacteria are so thin and transparent and are so unlike any bacteria previously described that I would have overlooked them if they had not possessed gas vacuoles, and I had not been looking for different forms of gas-vacuolate organisms. The squares derive buoyancy from their gas vacuoles and float at the brine surface.

Gas vacuoles are found exclusively in prokaryotic organisms and occur mainly in planktonic forms<sup>1,2</sup>. It has been proposed that, through providing or regulating buoyancy, they are important in positioning these organisms at particular depths in natural waters<sup>2</sup>. Surveys of freshwater lakes<sup>3,4</sup> suggest that gas-vacuolate bacteria are to be found in all lakes which are stable or stratified and I wondered whether this applied also to saline pools. Gas-vacuolate bacteria of the genus *Halobacterium* have been isolated from solar salt<sup>5</sup> and it has been speculated that their gas vacuoles buoy them to the surface of brine pools where the oxygen these obligate aerobes require is more readily available<sup>6</sup>. However, this has yet to be substantiated by field observations<sup>7</sup>.

I collected brine from a salt crust forming at the surface of a hypersaline pool in a sabkra (a coastal plain at sea level) just south of Nabq, on the Sinai Peninsula. The chemistry of the pool and its location are described in detail by Krumbein *et al.*<sup>7</sup>. Microscopic examination revealed abundant gas-vacuolate organisms. At least five morphologically distinct forms were present, and of these the square bacteria were most abundant. Using a Hawksley standard counting chamber, I estimated that there were  $7 \times 10^7$  squares per ml of liquid brine.

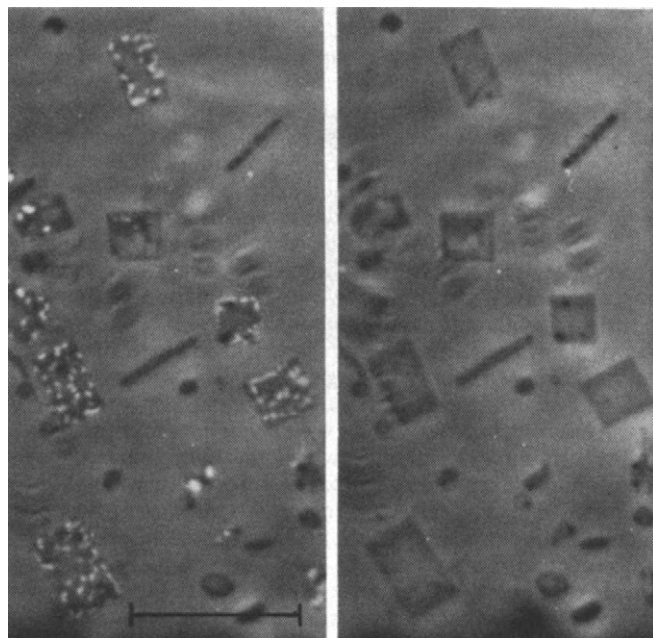
The presence of gas vacuoles in the various forms was demonstrated by comparing the appearance of individual cells before and after application of pressure (Fig. 1) as described previously<sup>3</sup>. Gas vacuoles are made up of minute hollow, proteinaceous structures called gas vesicles<sup>8</sup>. By virtue of their small size and the low refractive index of their contents, they scatter light very strongly and show as brilliant light objects against the dark ground of the cytoplasm under the phase contrast microscope. However, they can be made to collapse irreversibly by application of pressure and then they are no longer visible. This disappearance is diagnostic of gas vesicles—and the gas vacuoles they form—and identifies them unambiguously<sup>2,3</sup>. The presence of gas vacuoles in the squares firmly established their bacterial nature. Attempts to culture the organism were unsuccessful, but samples of brine were enriched for the square bacterium by centrifugation, as follows. A drop of the sample was mixed with the saline solution described below and placed in a haematocrit tube, forming a layer 18 mm deep. The tube was centrifuged at 227g for 100 min. The pressure generated at the base of the tube in these conditions was only 0.4 bars, too small to collapse a significant quantity of vesicles<sup>9</sup>. The buoyant, gas-vacuolate square bacteria rose to the meniscus from where they could be transferred by microsyringe. The squares also aggregated at the meniscus of the brine sample after leaving it to stand for two days. The squares which floated in contact with the coverslip of the counting chamber sank after application of pressures causing gas vacuole collapse. This showed that the square bacteria depended on their gas vacuoles for buoyancy.

The membranous appearance of the squares at first suggested that they might be the walls of other disintegrated bacteria to which gas vacuoles had remained attached. However, some of the sheets,  $>20 \mu\text{m}^2$ , were clearly too large for this. Moreover, the thickness of the sheets,  $0.2\text{--}0.5 \mu\text{m}$ , which could just be discerned from edge-on views, would be too much for a simple wall but just enough for a bacterium. This interpretation was supported by finding cells which were evidently in various stages of division (Fig. 2). The gas vacuoles were frequently concentrated around the edges of the squares and along the presumptive division lines. Undivided squares ranged in size from  $1.5$  to  $11 \mu\text{m}$  (Fig. 2). Some squares had a number of division lines (strictly speaking, planes). Their arrangement indicated that the division occurred in two planes alternating at right angles; this produced sheets divided like postage stamps. Sheets of eight squares were not uncommon and sheets of 16 were found with individual squares tearing free.

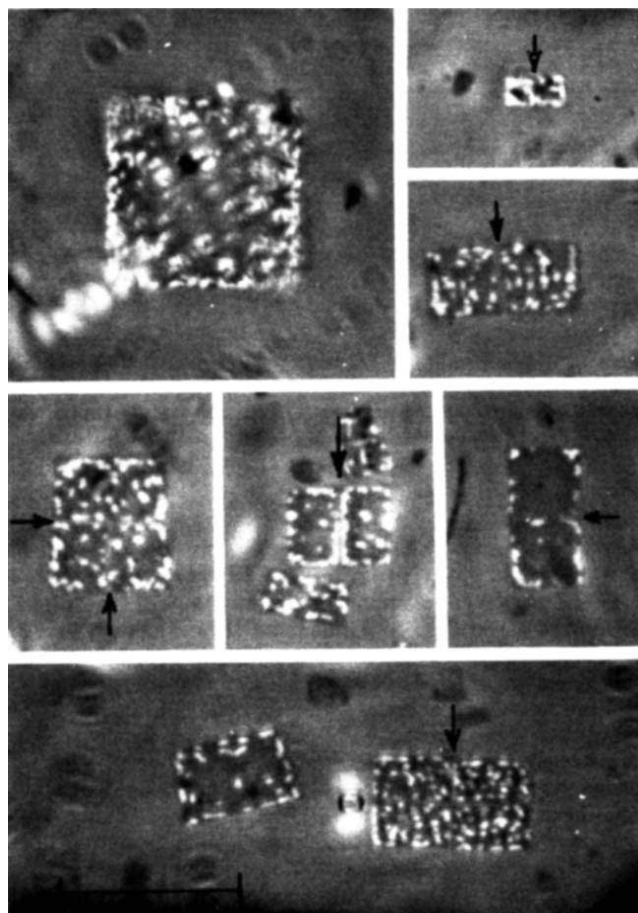
Electron microscopy was carried out on samples of brine enriched for the square bacteria. The gas vesicles, which could be seen in whole-mounted unstained preparations, were similar to those of other bacteria, cylinders with cone-shaped ends<sup>1,2</sup>. Metal shadowing showed that the surface of the square bacteria was covered in particles  $\sim 2 \text{ nm}$  across, arranged in a regular hexagonal lattice. This is similar to the arrangement observed in halobacteria<sup>10</sup> and suggests that, like them, the square bacteria belong to the group designated Archaeobacteria<sup>11</sup>. Another feature of this group, satisfied by both, is that they occur in extreme environments. However, the phylogenetic relationships of the square bacterium can be established only by analysis of material from axenic cultures.

The regular array into which the cell envelope particles are assembled presumably determines the shape of the square bacterium. It is postulated that such a shape is permitted only by the absence of turgor pressure in this organism.

The majority of known bacteria have shapes such as spheres, ellipsoids or cylinders which are suitable for withstanding internal pressure. However, if pressure were generated in the square bacterium the flat surfaces would tend to bow outwards, break or come apart at the joining edges. Because the squares are obviously stable and remain flat they must have a low turgor pressure inside them. Another extreme halophile with gas



**Fig. 1** Phase contrast light micrograph of a field of square bacteria seen before (on the left) and after (right) application of 5 bars pressure which causes the gas vacuoles to disappear. Scale bar  $10 \mu\text{m}$ .



**Fig. 2** Phase contrast light micrographs of the square bacterium illustrating the range of cell size. The light areas are gas vacuoles. Division lines, just detectable on the original print are indicated by the arrows. Scale bar,  $10 \mu\text{m}$ .

vacuoles, *Halobacterium* sp.<sup>12</sup> has no detectable turgor pressure<sup>13</sup> and may, in certain conditions, produce rather flattened cells<sup>10</sup>. (*Simonsiella* spp. also have flattened cells, arranged in filaments<sup>14</sup>, but nothing is known of their osmotic relationships.)

Cell turgor pressure can, in principle, be measured in any prokaryote which contains gas vacuoles. The constituent gas vesicles collapse irreversibly over a well-defined range of pressure. Inside the cell, turgor pressure contributes to the pressure required but if a non-penetrating solute, such as sucrose, is added to the suspending medium the turgor pressure is reduced and the pressure required to collapse the gas vesicles rises accordingly. Measurements on various freshwater prokaryotes suspended first in water and then in hypertonic sucrose solutions have revealed turgor pressures of mainly  $2\text{--}5 \text{ bars}$ <sup>13</sup>, and increases in such pressures over this range cause the cells to swell<sup>15</sup>. The unusual absence of turgor in halobacteria is evidently related to the high salt concentration of their surroundings<sup>13</sup>.

Accurate measurements of turgor pressure by the gas-vacuole method require small but homogeneous suspensions of the organism, though a rough estimate can be made from observations on the disappearance of gas vesicles in individual cells under the microscope. A  $4\text{-}\mu\text{l}$  sample of the brine was mixed with  $15 \mu\text{l}$  of  $1 \text{ M}$  sucrose dissolved in a salt solution containing  $\text{NaCl}$  ( $250 \text{ g l}^{-1}$ ),  $\text{KCl}$  ( $5 \text{ g l}^{-1}$ ),  $\text{MgSO}_4$  ( $10 \text{ g l}^{-1}$ ) and  $\text{CaCl}_2$  ( $0.13 \text{ g l}^{-1}$ ), the main mineral ingredients of a halobacterium culture medium<sup>16</sup>. A control sample was mixed with the same salt solution without sucrose. Drops of the mixtures were placed on Hawksley counting chambers, depth  $0.02 \text{ mm}$ , and a coverslip was positioned acentrically to provide an air gap to the channel surrounding the platform<sup>3</sup>. The bacteria were observed under phase contrast illumination using a  $\times 1,000$  oil immersion



objective and again after pressurising the slide at 1, 1.5, 2, 2.5, 3 and 10 bars. Individual gas vesicles could be seen but not separately resolved at this magnification. In both mixtures with and without sucrose a definite reduction in gas vesicle numbers per cell was seen after application of 1.5 bars, most of the vesicles had disappeared by 2.5 bars, and none remained beyond 3 bars. The lack of difference between the two samples with respect to the pressure required to collapse gas vesicles shows that turgor pressure was small or non-existent<sup>13</sup>.

In the absence of turgor pressure there is no obvious constraint on the shape of a bacterium and in theory any shape might be adopted, depending on the shape and arrangement of units which make up the cell wall. Whatever determines its shape apparently allows for some variation as occasionally triangular and trapezoid shapes are seen. These appear to be complete cells and not simply squares which have been folded or torn. The similarity in shape of the bacterium and the salt crystals with which it coexists is probably fortuitous.

The brine sample was collected on a visit kindly arranged by Professor Moshe Shilo and supported by the Royal Society. I thank Professor Wolfgang Krumbein for his geomicrobiological tour of Sinai and the brine pool, and Mr Ken Parkes for the electron microscopy.

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## Cytoplasmic incompatibility in natural populations of a mosquito, *Culex pipiens* L.

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When two strains of *Culex pipiens* (s.l.) of different geographical origin are cross-mated, the cross is frequently sterile in one or both directions<sup>1</sup>. Such incompatibility is said to be cytoplasmic because the crossability of a strain is determined by its maternal lineage<sup>2</sup>. The incompatibility is caused in some way by infection with a rickettsia-like bacterial symbiote, as removal of the symbiote abolishes the incompatibility<sup>3</sup>. Incompatibility has not been observed in crosses of American strains of *C. pipiens*<sup>4–7</sup>. On the other hand, most workers in other parts of the world who have crossed *C. pipiens* strains have noticed incompatibility<sup>7–14</sup>, although there are no reports of incompatible egg rafts being collected in the field. We now report incompatibility in crosses of sympatric American strains of *C. pipiens* and the collection of incompatible egg rafts in the field.

On 4 July 1978, an egg raft of *Culex pipiens quinquefasciatus* Say was collected in Malibu, Los Angeles County, California. Its appearance suggested that the egg raft had been laid by a female which mated with an incompatible male; about 80% of the eggs showed evidence of embryonic development and few of these had developing setae visible, indicating that the embryos died early in development. About 20% of the eggs did not show

evidence of embryonic development, and these were scattered throughout the raft rather than being in a group at one end. Three similar rafts have been collected from the same breeding place, on 15 July, 24 July and 22 August 1978. So far, 48 egg rafts have been examined from this breeding place, of which 43 hatched normally, 4 were apparently incompatible, and 1 showed no embryonic development at all, which indicates that it was probably laid by an unseminated female.

**Table 1** Crossing types of egg rafts collected in southern California

| Locality         | Crossing type* |   |   | Total |
|------------------|----------------|---|---|-------|
|                  | A              | B | C |       |
| Malibu           | 31             | 1 | 1 | 33    |
| West Covina      | 8              | 1 | 0 | 9     |
| West Los Angeles | 7              | 0 | 1 | 8     |
| Venice           | 3              | 0 | 0 | 3     |
| Mar Vista        | 15             | 0 | 1 | 16    |
| Total            | 64             | 2 | 3 | 69    |

\* A, Reciprocally compatible with Californian crossing type; B, females incompatible with males of Californian crossing type, reciprocal cross compatible; C, males incompatible with females of Californian crossing type, reciprocal cross compatible.

After the second incompatible egg raft was observed (15 July), all egg rafts collected were taken to the laboratory, allowed to hatch, the larvae reared, and the adults reciprocally crossed with a mongrel *C. pipiens* laboratory strain of Californian crossing type (here designated crossing type A). Adults were placed in small cages (1.5-l capacity) and females fed on human or mouse blood. Thirty-three progenies have now been successfully tested; 31 were compatible when crossed in either direction with the laboratory strain. The females of one progeny (crossing type B) were incompatible when crossed with males of the laboratory strain, although the reciprocal cross was fertile. The males of another progeny (crossing type C) were incompatible with females of the laboratory strain, although the reciprocal cross was fertile.

In three other localities in greater Los Angeles (West Covina, West Los Angeles, Mar Vista), mixed populations of crossing types have also been found (Table 1), although incompatible egg rafts have not yet been taken in the field in these locations.

A laboratory strain was developed from sibling females of crossing type C which had been crossed with males of type A. In the F<sub>4</sub> of this strain, 10 of 60 egg rafts examined were incompatible. In the F<sub>5</sub>, 2 of 39 examined were incompatible. This is the first report of incompatibility between individuals from a common female ancestor, although in this case the incompatible individuals may have been third or fourth cousins.

Variation in crossing type in the progeny of a female has been reported twice before<sup>15,16</sup>, but incompatibility between individuals derived ultimately from a single female has not been reported. The present data suggest that there may be a mixed population of crossing type determinants in a female and these may segregate in her progeny to produce incompatibility in crosses between siblings. The cost of this incompatibility may be judged by the fact that 4 of 47 (8.5%) egg rafts of inseminated females taken at the Malibu breeding place failed to produce larvae.

It has been argued that two incompatible crossing types cannot exist for long in the same population because one type will eliminate the other in a fairly short time<sup>17</sup>. This argument is based on the assumption that each crossing type is inviable. It now seems probable that an individual mosquito may contain symbiotes producing two different and incompatible crossing types. A female may thus produce offspring which are of more than one crossing type and some offspring may be incompatible when mated with their siblings. Incompatibility in this case is a less effective selective agent for ridding the population of the incompatible crossing type. Incompatibility may thus persist in a natural population for a long time even though it is selected

against when expressed. The consequence for the host population is reproductive waste and for the symbiotes a reduction in fitness of their hosts.

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## Influence of H-2 complex on acquired resistance to *Leishmania donovani* infection in mice

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Innate susceptibility to *Leishmania donovani* infection in mice (measured over 2-4 weeks) is under the control of a single autosomal gene (*Lsh*) segregating for incompletely dominant resistant (*r*) and recessive susceptible (*s*) alleles<sup>1</sup>. This locus maps<sup>2</sup> away from the known histocompatibility loci to a position between the centromere and *Id-1* on the chromosome 1 of the mouse. Amongst homozygous recessive *Lsh*<sup>s</sup> strains of mice two patterns of recovery are observed<sup>3</sup> when the course of infection is followed over a longer term. In some strains a dramatic fall in parasite numbers with histological liver damage occurs while other strains maintain immense parasite loads for up to two years involving mononuclear phagocytes throughout the body. We now present evidence which suggests that this difference in long-term response is largely controlled by a gene(s) within, or closely adjacent to, the major histocompatibility complex (H-2) of the mouse.

Initially C57BL/10ScSn and B10.D2/n congenic mice plus their F<sub>1</sub>, F<sub>2</sub> and backcross progeny were infected with *L. donovani* and the course of their infections followed over 130 days. In the parental strains all of the B10.D2/n mice maintained heavy infections over the 130 days while liver parasite burdens in C57BL/10ScSn mice began to fall by day 50. All of the F<sub>1</sub> and backcross to B10.D2/n mice followed the B10.D2/n pattern of long-term high infections. F<sub>2</sub> and backcross to C57BL/10ScSn mice appeared to be segregating for high and low parasite burdens by days 85 and 130. When graphed according to H-2 haplotype (Fig. 1), H-2<sup>d/d</sup> and H-2<sup>b/d</sup> mice always maintained long-term heavy infections while H-2<sup>b/b</sup> mice showed a reduction in parasite load. Although the time course for the early stage of this recovery differed slightly between experiments, a significant difference between H-2<sup>b/b</sup> and H-2<sup>b/d</sup> mice and between H-2<sup>b/b</sup> and H-2<sup>d/d</sup> mice was always observed 85 days

after infection. H-2<sup>b/d</sup> and H-2<sup>d/d</sup> mice never differed significantly during the 130 days over which infections were followed. We concluded, therefore, that a gene(s) within, or closely adjacent to, H-2 controls an acquired resistance mechanism which behaves as a recessive trait and allows some innately susceptible mice to recover from an initially heavy infection of *L. donovani*. We propose *Rld-1* (recovery from *L. donovani*) as the provisional designation for this locus following the pattern laid down for H-2 linked genes controlling resistance to Gross leukaemia virus (*Rgv-1*)<sup>6</sup>, Friend virus leukaemogenesis (*Rfv-1*)<sup>7</sup>, and radiation leukaemia virus (*Rrv-1*)<sup>8</sup>. The two phenotypic patterns of long term infection we refer to as 'cure' and 'noncure'.

Following this discovery, four other H-2 haplotypes (*q*, *f*, *s* and *r*) on a B10 genetic background were tested for long-term response to *L. donovani* infection (Figs 2 and 3). The *q* (strain B10.G) mice showed the typical 'noncure' response, never differing significantly from control B10.D2/n and (B10.D2/n × C57BL/10ScSn)F<sub>1</sub> mice. The *f* (strain B10.M) mice also showed

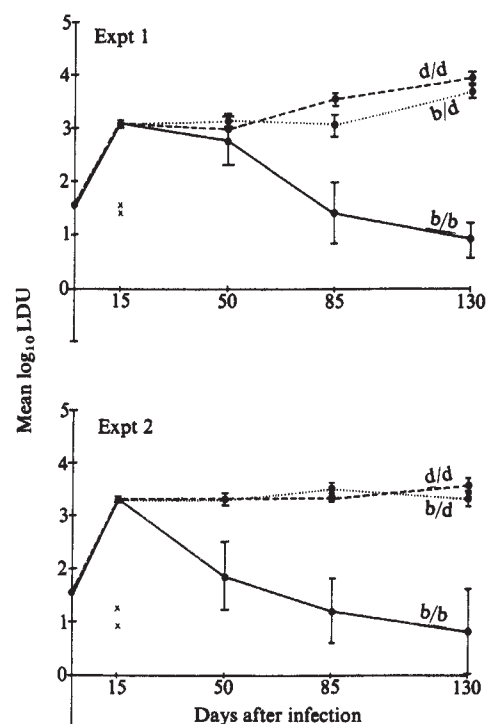
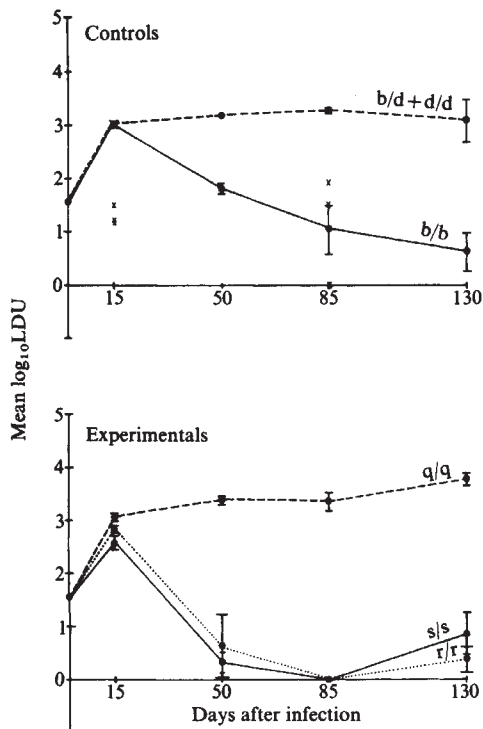


Fig. 1 For each experiment, groups of 10 mice from strains C57BL/10ScSn and B10.D2/n and from their F<sub>1</sub>, F<sub>2</sub> and backcross progenies (London Hospital Immunology Department) were infected with 10<sup>7</sup> amastigotes *L. donovani* as described by Bradley and Kirkley<sup>3</sup>. Two mice from each group were killed on days 15, 50 and 85 following infection and four mice from each group on day 130. The number of parasites per 500 liver cell nuclei were counted from Giemsa-stained impression smears and the results expressed as 'Leishman Donovan units' (LDU)<sup>4</sup>. All mice except those killed on day 15 were individually H-2 haplotyped using microcytotoxicity testing<sup>5</sup> with two NIH private specificity antisera for each end (K and D) of H-2. Results graphed are mean log<sub>10</sub>LDU counts (±1 s.e.) against days after infection for mice grouped according to the haplotypes: homozygous *b/b*, heterozygous *b/d*, and homozygous *d/d*. The day 0 points on the graphs represent approximately 70% uptake of the initial dose of amastigotes by the liver Kupffer cells found in many strains of mice by Bradley and Kirkley<sup>3</sup>. Since C57BL/10ScSn and B10.D2/n mice do not differ significantly in their day 15 parasite burdens<sup>1</sup>, counts for all mice were pooled to obtain the day 15 points on the graphs. This is further justified by the very small standard errors (0.05 and 0.04) obtained for the 12 mice killed on day 15 in each experiment. Crosses (x) represent individual counts from control homozygous *Lsh*<sup>s</sup> mice (C3H/He-mg/Ola and C57L) included in each experiment. Student's *t*-tests were used to test when differences in mean parasite loads between mice of different haplotypes became statistically significant. In both experiments *b/b* mice had significantly lower parasite loads than either *b/d* or *d/d* mice on days 85 ( $P < 0.02$ ) and 130 ( $P < 0.001$ ). In the second experiment the day 50 counts for *b/b* mice were already significantly lower ( $P < 0.02$ ). Mice of *b/d* and *d/d* haplotypes never differed significantly from each other.

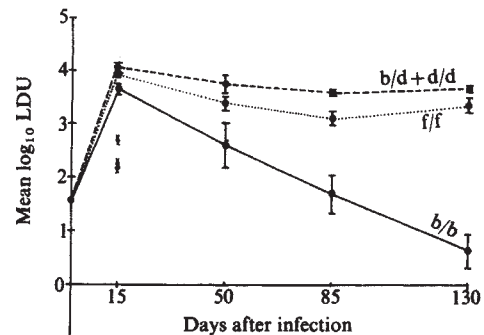




**Fig. 2** Mice with *q*, *s* and *r* H-2 haplotypes on a B10 genetic background were tested for long-term response to *L. donovani* by infecting 17 B10.G/Ola, 15 B10.S/Ola and 15 B10.RIII(71NS)/Ola mice. For the B10.G strain, four mice were killed on each of days 15, 50 and 130 and five mice on day 85. For B10.S and B10.RIII strains, three mice were killed on day 15 and 4 mice on each of the days 50, 85 and 130. Control 'cure' and 'noncure' mice included 12 C57BL/10ScSn (Ola and Nuffield Foundation), 6(B10.D2/n × C57BL/10ScSn)F<sub>1</sub> and 4 B10.D2/n/Ola mice. Since *d/d* (B10.D2/n) and *b/d* (F<sub>1</sub>) mice do not differ significantly in their long-term response (Fig. 1), mice from these two groups were pooled as the 'noncure' control. Only three control mice were killed on the early days of infection allowing four 'cure' and four 'noncure' mice to be killed on each of days 85 and 130. Results graphed are mean log<sub>10</sub>LDU counts (±1 s.e.) for each group of mice against days after infection. Mean log counts for B10.S and B10.RIII mice did not differ significantly throughout the experiment. Neither did B10.G differ from control 'noncure' mice. B10.S and B10.RIII mice had significantly lower mean log counts on day 15 than both B10.G ( $P < 0.05$ ) and the control 'cure' and 'noncure' mice ( $P < 0.05$ ). Mean day 15 LDU counts for these two strains were still, however, 28-fold higher than mean LDU counts from homozygous *Lsh*<sup>+</sup> mice (C3H/He-mg/Ola mice indicated by ×). Mean log counts also declined earlier in these two strains than in control 'cure' mice (day 50,  $P < 0.01$ ). This difference was no longer present on days 85 and 130. The levels of parasitaemia maintained in 'cure' mice at this stage in infection falls close to or below the threshold level for finding one parasite on an impression smear<sup>9</sup> and any slight differences between days and between strains are magnified by the log scale required to graph the extreme differences in parasite loads between 'cure' and 'noncure' mice. Hence we place no significance on the apparent increase in parasite loads between days 85 and 130 in B10.S and B10.RIII mice. These low levels of parasitaemia are of the same order of magnitude as those maintained in innately resistant (*Lsh*<sup>+</sup>) mice (this experiment day 85 and Bradley and Kirkley<sup>3</sup>).

an essentially 'noncure' response. Although logLDU counts for these mice were slightly lower than control 'noncure' mice throughout the experiment, the difference only reached statistical significance ( $P < 0.01$ ) on day 85. Day 130 counts did not differ significantly between *f* haplotype and control 'noncure' mice. A significant difference was, however, observed between *f* haplotype and control 'cure' mice on days 85 ( $P < 0.01$ ) and 130 ( $P < 0.001$ ). The *s* (strain B10.S) and *r* (strain B10.RIII) haplotype mice displayed an acquired resistance mechanism similar to, but effective earlier than, that observed in C57BL/10ScSn *b* haplotype mice. Day 15 parasite levels in these two strains indicated that this acquired resistance mechanism may be operating sufficiently early to modify *Lsh* gene control of innate susceptibility. Parasite burdens were still, however, 28-fold higher than those observed in *Lsh*<sup>+</sup> mice.

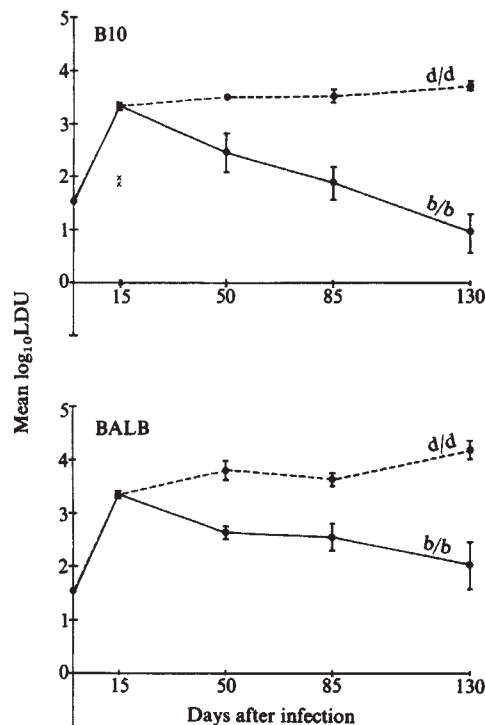
Our finding that 'cure' behaves as a recessive trait in H-2<sup>b/d</sup> mice is unusual in that most H-2 linked *Ir* genes show dominant responsiveness<sup>10</sup>. H-2 linked loci which do control apparent recessive immune responsiveness<sup>7,11-13</sup> do not follow the same distribution of haplotypes between 'responder' and 'nonresponder' strains as we have shown between 'cure' and 'noncure' strains. It is unlikely, therefore, that we are dealing with a known H-2 linked gene. Increasing evidence suggests that apparent recessive immune responsiveness may, in some cases, be due to dominant immune suppressor (*Is*) genes<sup>14-16</sup>. These *Is* genes exhibit many genetic similarities with *Ir* genes and map alongside them in the I region of the H-2 complex<sup>15,16</sup>. In other cases<sup>7,13</sup> apparent recessive immune responsiveness is controlled by genes mapping away from the I region of H-2 indicating a mechanism which may be quite independent of the *Ir* gene system<sup>17</sup>. If recovery from *L. donovani* infection were observed in other 'cure'/'noncure' heterozygotes, an immune suppression mechanism rather than recessive immune responsiveness would be favoured. We may thus be dealing with more than one locus within H-2, one controlling immune responsiveness and one controlling immune suppression. Varying rates of recovery between different 'cure' haplotypes could then be explained by a gene-dosage hypothesis. Our current examination of various heterozygous haplotypes as well as our attempts to map *Rld-1* within H-2 using recombinant haplotype strains should resolve these questions.



**Fig. 3** Mice with the *f* H-2 haplotype on a B10 genetic background were tested for long-term response to *L. donovani* by infecting 15 B10.M/Ola mice. Three mice were killed on day 15, and four mice on each of days 50, 85 and 130. Three control 'cure' C57BL/10ScSn mice and three control 'noncure' (B10.D2/n × C57BL/10ScSn)F<sub>1</sub> mice were killed on each of days 15, 50 and 85, and four mice from each group on day 130. Three control 'noncure' B10.D2/n/Ola mice were also killed on day 85. Results graphed are mean log<sub>10</sub>LDU counts (±1 s.e.) for each group of mice against days after infection. Mean log counts for B10.M mice were slightly lower than control 'noncure' mice throughout the experiment but differed significantly only on day 85 ( $P < 0.01$ ). Control 'cure' mice had significantly lower mean log counts than both B10.M and control 'noncure' mice on days 85 ( $P < 0.01$ ,  $P < 0.001$ ) and 130 ( $P < 0.001$  in both cases). Crosses (×) represent individual counts from control homozygous *Lsh*<sup>+</sup> (DBA/2) mice.

Finally, we have attempted to determine whether the acquired resistance mechanism operates for the same H-2 haplotypes on an alternative genetic background. Although chosen because BALB was the only other genetic background carrying the *Lsh*<sup>+</sup> allele, BALB H-2 congenic strains did seem a particularly good choice for this experiment as BALB/c mice were already known to maintain chronic heavy infections involving mononuclear phagocytes throughout the body. BALB/c (*d* haplotype) and BALB/B (*b* haplotype) mice were tested for long-term response to *L. donovani* infection (Fig. 4). The acquired resistance mechanism of the *b* haplotype did appear to operate on the BALB genetic background.

Although marked H-2 restrictions have been demonstrated in the adoptive transfer of resistance to both bacteria<sup>18</sup> and viruses<sup>19</sup>, the role of H-2 linked genes in determining actual resistance to infecting agents has previously only been defined for viruses. The present investigation was made possible only by our previous understanding of the genetic control of acute, or innate, immunity to *L. donovani* infection in mice, allowing this



**Fig. 4** Mice with *b* and *d* H-2 haplotypes on a BALB genetic background were tested for long-term response to *L. donovani* by infecting eight BALB/B/Ola and 10 BALB/c/Ola mice. For the BALB/B strain, two mice were killed on day 50 and three mice on each of days 85 and 130. For the BALB/c strain, one mouse was killed on day 15 and three mice on each of days 50, 85 and 130. Control 'cure' and 'noncure' mice included 15 C57BL/10ScSn (Ola and Nuffield Foundation) mice and 13 B10.D2/n (Ola and LSHTM) mice. Three mice from each control strain were killed on day 15, 2 on day 50, and five on day 85. Five C57BL/10ScSn and three B10.D2/n mice were killed on day 130. [The low number of BALB mice used in this experiment was due to a shortage in supply and this experiment is being repeated.] Although only one BALB/c mouse was tested here, BALB/c mice are known not to differ significantly in their day 15 mean log counts from C57BL/10ScSn and B10.D2/n mice<sup>1</sup>. We have tested this again many times (unpublished data) and have also subsequently tested the day 15 parasite levels in BALB/B mice. These do not differ significantly from BALB/c, C57BL/10ScSn or B10.D2/n mice. The day 15 points on the graphs therefore represent the mean log count ( $\pm 1$  s.e.) for the seven *Lsh*<sup>+</sup> (CBA/Ca/Ola) mice are indicated by a cross (x). Mean log counts obtained on days 50, 85 and 130 did not differ significantly between BALB/B and C57BL/10ScSn mice or between BALB/c and B10.D2/n mice. Mean log counts for BALB/B mice were significantly lower than BALB/c and B10.D2/n mice on days 50, 85 and 130 ( $P < 0.02$  in each case), as were C57BL/10ScSn mean log counts ( $P < 0.01$  in each case).

to be standardised in experiments analysing recovery and acquired immunity. Further study of the mechanisms involved in both the innate and acquired responses will be important to our understanding of this and other related human diseases, particularly in view of the recent implications of HLA linked susceptibility to leprosy<sup>20,21</sup> and malaria<sup>22</sup> in man. The mouse model allows a more precise correlation with major histocompatibility type, more scope for genetic manipulation, and a more direct access to the mechanisms involved.

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## Formation of SV40 specific and H-2 restricted target cell antigen by somatic cell fusion

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Cytotoxic T lymphocytes (CTLs) to syngeneic Simian virus-40 (SV40)-transformed target cells lyse only SV40-transformed cells which are identical to the killer cells at the K and/or D loci of the H-2 complex in mice<sup>1,2</sup>. In fact, CTLs have been shown to require autologous H-2 antigen expression for target cell lysis in a variety of syngeneic systems<sup>3</sup>. The dual specificity of these lymphocytes could indicate that both virus-coded and H-2 gene products are recognised and that interaction between viral and H-2 antigens on the tumour cell surface may be required to form the target antigen. However, serologically detectable SV40-specific antigens have not been found on the transformed cell surface. In addition, antisera to viral proteins do not block T-cell mediated lysis (CML) of syngeneic transformants<sup>4</sup>. If an SV40-specific moiety does not actually exist at the cell membrane, T-cell recognition might occur via an 'altered self' antigen where the virus codes for some activity which alters the H-2 antigens at the level of transcription or translation. To test this hypothesis the following experiments were designed: target cells were 'manufactured' by polyethylene glycol (PEG)-induced fusion of two cell lines; one being syngeneic to the CTL but not SV40 transformed and the other possessing the SV40 genome but differing from the effectors at the H-2 locus. The resulting multinucleated cells (heterokaryons) were then used as targets in the CML assay. Membrane components of heterokaryon cells have been shown to intermix<sup>5</sup>. In the present experiments we reasoned that if the target antigen is an H-2K or H-2D molecule which has been altered in transcription or translation, then simple mixing of membrane components would not lead to target antigen formation and cell lysis. If, on the other hand, separate SV40 induced and H-2 molecules are required for recognition then heterokaryon formation should lead to target antigen expression. Results described below support the latter hypothesis. Furthermore, these results show the simple mixing of cellular components is sufficient for a recognisable target antigen to form, and that protein synthesis is not required.

Cell lines which were chosen as parents for fusion were mixed together and plated on 100-m tissue culture dishes (Falcon). The density at which each cell line was used was determined by the growth rate of that line; slower growing cells were plated at larger numbers than fast growing cells (see Table 1). Cells were ready for fusion with PEG when light monolayers were



**Table 1** Fusion of cell lines of various antigen specificities

| Target cells                           |                                | % Specific lysis |                     |                                 |                                                                            |
|----------------------------------------|--------------------------------|------------------|---------------------|---------------------------------|----------------------------------------------------------------------------|
| <sup>51</sup> Cr labelled × unlabelled | Cell densities used for mixing | PEG              | % Fusion efficiency | C3H anti-H-2 <sup>b</sup> /SV40 | (H-2 <sup>d</sup> × H-2 <sup>b</sup> )F <sub>1</sub> anti-H-2 <sup>k</sup> |
| H-2 <sup>k</sup> /SV40                 | 1.2 × 10 <sup>6</sup>          | —                | —                   | 74%                             | 59%                                                                        |
| H-2 <sup>b</sup> /SV40                 | 1.2 × 10 <sup>6</sup>          | —                | —                   | 1%                              | —4%                                                                        |
| H-2 <sup>d</sup> /SV40                 | 1.2 × 10 <sup>6</sup>          | —                | —                   | 10%                             | —6%                                                                        |
| H-2 <sup>k</sup>                       | 1.8 × 10 <sup>6</sup>          | —                | —                   | 3%                              | 73%                                                                        |
| H-2 <sup>k</sup> /SV40*                | —                              | —                | —                   | 42%                             | ND                                                                         |
| H-2 <sup>k</sup> /SV40*                | —                              | +                | —                   | 47%                             | ND                                                                         |
| H-2 <sup>b</sup> /SV40*                | —                              | —                | —                   | 2%                              | ND                                                                         |
| H-2 <sup>b</sup> /SV40*                | —                              | +                | —                   | 1%                              | ND                                                                         |
| H-2 <sup>k</sup> *                     | —                              | —                | —                   | 5%                              | ND                                                                         |
| H-2 <sup>k</sup> *                     | —                              | +                | —                   | 7%                              | ND                                                                         |
| H-2 <sup>b</sup> /SV40                 | H-2 <sup>k</sup> /SV40         | +                | 47%                 | 42%                             | 20%                                                                        |
| H-2 <sup>d</sup> /SV40                 | H-2 <sup>k</sup> /SV40         | +                | 45%                 | 46%                             | 34%                                                                        |
| H-2 <sup>k</sup> /SV40                 | H-2 <sup>k</sup>               | +                | 59%                 | 37%                             | ND                                                                         |
| H-2 <sup>k</sup>                       | H-2 <sup>b</sup> /SV40         | +                | 43%                 | 21%                             | 21%                                                                        |

Cells were fused using the procedure of Davison and Gerald<sup>6</sup> with slight modifications. Parent cells for fusion were first labelled for 12 h with 100  $\mu$ Ci of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (200–500 Ci g<sup>-1</sup>, NEN, <sup>51</sup>Cr), then washed three times in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine and 1.0% antibiotic/antimycotic mixture (Gibco, DME 10), and collected using 0.02% trypsin. Tissue culture dishes (100 mm) were then inoculated with the two parent cell types at the cell densities given and incubated in DME 10 at 37 °C, 5% CO<sub>2</sub> and 95% air. After 12 h the medium was aspirated off and the dishes were washed one time with phosphate-buffered saline (PBS). The cells were then covered with 4 ml of fusing solution (47% polyethylene glycol 6000, Baker, 10% dimethyl sulphoxide, in modified Eagles medium). After 1 min at room temperature the fusing solution was thoroughly rinsed from the dishes with PBS. Dishes were then incubated at 37 °C in DME 10. After 2 h cells were removed with trypsin, washed once with modified Eagles medium containing fetal calf serum, glutamine and antibiotics as above (MEM 10) and an aliquot was removed to determine the efficiency of fusion. One drop of cell suspension was added to one drop of 0.1% acridine orange-PBS solution and cells were immediately viewed under a Leitz fluorescence microscope. The per cent of the total nuclei present in multinucleated cells was determined and expressed as

$$\% \text{ Efficiency of fusion} = \frac{\text{No. nuclei present in multinucleates}}{\text{Total no. of nuclei}} \times 100$$

The fused cell population was expected to contain parent cells, monokaryons and heterokaryons. To reduce the parent cell populations and to minimise homokaryon formation cells were subjected to PEG treatment in conditions which insured maximum fusion. In these conditions most multinucleated cells were found to contain more than 2 and up to 20 or more nuclei. The <sup>51</sup>Cr-release assay was performed in triplicate using effectors generated in a secondary *in vitro* sensitisation against H-2<sup>k</sup>/SV40 cells as previously described<sup>7</sup> or against allogeneic spleen cells. At the end of the incubation period (6 h) aliquots were removed from assay wells and counted in Beckman Gamma 4000 counter. Results were calculated and are expressed as follows:

$$\% \text{ Specific lysis} = \frac{E - C}{T} \times 100, \text{ where}$$

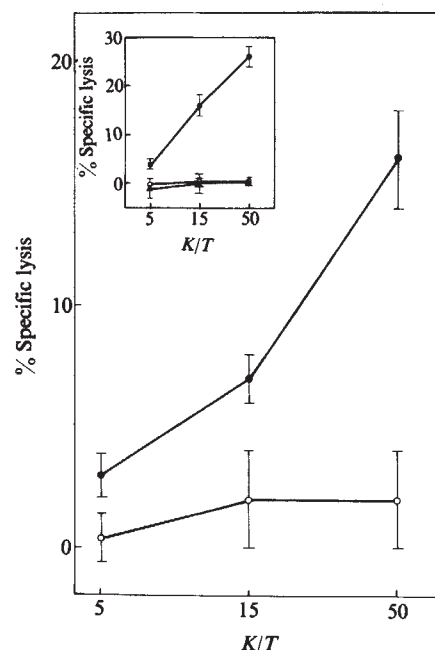
$E$  = <sup>51</sup>Cr released in the presence of sensitised spleen cells;  $C$  = <sup>51</sup>Cr released in the presence of non-sensitised spleen cells and  $T$  = total <sup>51</sup>Cr released by addition of 1 M HCl. Ratio of effector cells to target cells = 50:1. Cell lines are designated as follows: PSC3H (H-2<sup>k</sup>/SV40), PSSW (H-2<sup>b</sup>/SV40), SVBALB (H-2<sup>d</sup>/SV40), MM5 (H-2<sup>k</sup>). Mouse strains were C3H/HeJ and (B10.D2 × C3H.SW)F<sub>1</sub>.

\* These experiments were run separately and the per cent specific lysis is reported for an effector: target cell ratio of 15:1.

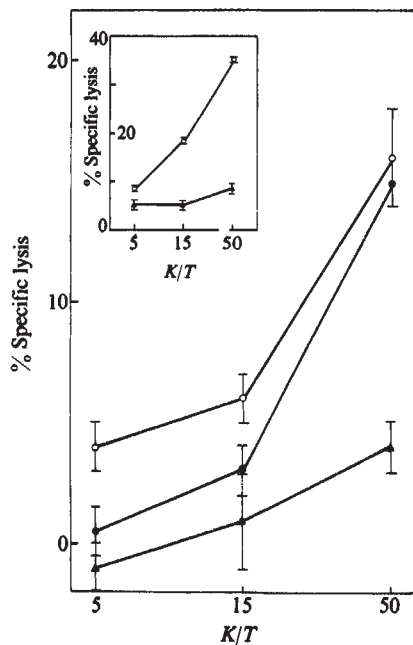
achieved. Cells were collected 2h after fusion and the efficiency of the fusion procedure was determined (for details see Table 1).

The efficiency of fusion was usually found to be between 50 and 80% and was probably dependent on the density and arrangement of cells on any particular tissue culture dish at the time of PEG treatment. After fusion with PEG, cells were prepared for incubation with CTLs to determine if target antigens were distinguishable on the heterokaryon cell surfaces. C3H/He effector T cells, specific for H-2<sup>k</sup>/SV40 antigens, were elicited and a <sup>51</sup>Cr-release assay was performed as previously described<sup>1</sup> (Fig. 1). The results of the CML assay indicated that CTLs were able specifically to lyse at least some cells in the PEG-treated cell population. The parent cells themselves were not lysed by the effectors as they lacked either SV40 transformation or the H-2<sup>k</sup> antigen. Furthermore, the per cent lysis of parent cells which had been subjected to the fusion procedure did not change significantly from the untreated controls. These data indicated that the heterokaryon cells had acquired the target antigen which neither parent by itself possessed and that PEG treatment alone did not cause binding and fusion of effector cells with 'incorrect' target cells.

Numerous reports have indicated that parental cells comprising heterokaryons are capable of inducing various activities in one another<sup>8</sup>. In fact, the SV40 T antigen has been shown to migrate in heterokaryons from the SV40-transformed nucleus to the nucleus of the normal parent<sup>9</sup>. Therefore, to confirm the hypothesis that target antigen formation involved simple mixing of two or more components already present in the transformed cell it was necessary to show that the SV40-transformed parent cells were not directing the non-SV40 cells to alter their 'self-antigens' at the levels of transcription or translation. It was predicted that if the target antigen on an SV40-transformed syngeneic cell was a product of freely moving moieties, then *de*



**Fig. 1** CML of heterokaryon target cells by anti-H-2<sup>k</sup>/SV40 restricted effectors at various killer to target ratios. Target cells are mixtures of H-2<sup>k</sup> and H-2<sup>b</sup>/SV40 cells fused with PEG (●), and H-2<sup>k</sup> and H-2<sup>b</sup>/SV40 cells not subjected to PEG treatment (○). All cells (including controls) were labelled at the time of mixing with 100  $\mu$ Ci <sup>51</sup>Cr per 100 mm dish. Cells were collected 2 h after PEG fusion with trypsin and used as targets in the <sup>51</sup>Cr-release CML assay (see Table 1). Insert: lysis of control targets by anti-H-2<sup>k</sup>/SV40 effectors: H-2<sup>k</sup>/SV40 (●); H-2<sup>k</sup> (○); and H-2<sup>b</sup>/SV40 (▲).



**Fig. 2** Lack of a requirement for *de novo* protein synthesis in target antigen formation on heterokaryon target cells. Immediately after PEG fusion cells were incubated either in the absence (DME 10–cyclo) or presence of 25 mM cycloheximide (DME 10 + cyclo). After 2 h cells were collected and aliquots removed for treatment with papain. Cells ( $2.5 \times 10^6$ ) in 0.5 ml were added to a solution containing 0.01 M cysteine, 50  $\mu$ g DNase and 5 mg of papain. The volume was brought to 5 ml with DME 10 (with or without cycloheximide) and the tubes were incubated at 37 °C, 5% CO<sub>2</sub> and 95% air. After 60 min cells were centrifuged at 100g for 10 min, washed three times with DME 10  $\pm$  cyclo and prepared for use in the <sup>51</sup>Cr-release assay. H-2<sup>k</sup> and H-2<sup>b</sup>/SV40 cells were fused and treated with cycloheximide (●); papain (○); or cycloheximide and papain (▲). Effector cells in the CML assay were H-2<sup>k</sup>/SV40 restricted. Insert: H-2<sup>k</sup>/SV40 cells were treated with papain (○), or cycloheximide and papain (▲) along with the experimentals.

*de novo* protein synthesis should not be required for target antigen formation in the heterokaryon cell. To address this point cycloheximide, an inhibitor of translation, was added to the incubation medium immediately after fusion with PEG (Fig. 2). The efficiency with which cycloheximide inhibited protein synthesis necessary for target antigen formation was tested by treating target cells with papain before the <sup>51</sup>Cr-release assay. Treatment with papain rendered the target cells refractile to lysis by the effectors probably by cleaving off part or all of the target antigen from the surface of the cell. Normally the SV40 target cell will synthesise more antigen and become susceptible to lysis by about 2 h (data not shown). Addition of cycloheximide effectively inhibited the reappearance of the target antigen following papain treatment both on the control targets (see insert Fig. 2) and on the heterokaryon cell population (Fig. 2). Although cycloheximide efficiently inhibited resynthesis of target antigens from the papain-treated cells, it had little effect on the 'manufacture' of target antigens on heterokaryons. Lysis of heterokaryon cell populations with or without cycloheximide by the anti-H-2<sup>k</sup>/SV40 effectors was very similar.

These results are most consistent with the hypothesis that mixing of virus-induced and H-2 moieties is sufficient for target antigen formation in SV40-transformed cells. The data do not support any hypothesis in which the target antigen is produced before or during translation. Our results are similar to those seen by Sugamura *et al.* with T-cell mediated lysis of syngeneic Sendai virus-infected cells, where fusion of inactivated virus into the cell membrane was also sufficient to produce the target antigen<sup>10</sup>.

Although these findings indicate that an 'altered-self' antigen is not produced by virus-directed transcriptional or translational modification of H-2 antigens, they do not rule out post-translational alterations. For example, the SV40-coded nuclear T antigen has been reported to have protein kinase activity<sup>11</sup>, so that phosphorylation of H-2 molecules could produce the T-cell

target antigen. Alternatively, T-cell recognition could require the formation of complexes between H-2 antigens and a virus-coded moiety on the cell surface. Finally, it remains possible that no interaction between virus and H-2 molecules is required, but that recognition is mediated via two separate receptors on the T cell for which only the proximity of molecules on the target cell surface is necessary.

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## Chromosome abnormalities of leukaemic B lymphocytes in chronic lymphocytic leukaemia

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Chromosome analyses have revealed abnormalities in many cases of lymphoma, non-lymphocytic leukaemia and acute lymphoblastic leukaemia. Most studies on chronic lymphocytic leukaemia (CLL), however, have shown normal karyotypes<sup>1–4</sup> although chromosomal abnormalities have been reported<sup>5,6</sup>. In most of these studies specific surface marker analyses on leukaemic lymphocytes have not been carried out. It has been difficult to establish whether the metaphases seen in the majority of the CLL studies were from normal or from leukaemic cells. Recently, in one of our laboratories special interest has centred on cases of CLL with monoclonal serum protein bands; in two such cases with immunoglobulin M (IgM) bands, it was demonstrated with idiotype antisera against the serum IgM band that the leukaemic lymphocytes were the precursors of plasma cells responsible for the monoclonal serum IgM<sup>7,8</sup>. In addition, the leukaemic lymphocytes of these patients could be stimulated *in vitro* to divide and to differentiate to plasma cells<sup>9</sup>. Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines were obtained from the two patients. With the idiotype determinants as leukaemia specific markers, certain of these lines were shown to be derived from leukaemic cells<sup>9</sup>. Thus, rapidly proliferating populations of cells, which could be identified with certainty as being of CLL origin, were available for chromosome analysis. In the present study, conventional G- and Q-banding techniques for karyotypic analysis were carried out on the leukaemic lymphocytes induced to divide *in vitro* and on the cells of the leukaemic lymphoblastoid lines. Aneuploid karyotypes were found in the leukaemic B cells of both patients.

In the first case, Se, multiple cell lines were obtained<sup>9</sup> before the patient received chemotherapy or radiation. One of these lines, SeD, derived from splenic tissue, was shown to be derived





**Fig. 1** Partial karyotypes showing all the abnormalities detected in the leukaemic cells from patient Se (G-banding). Cell line SeD, which had been derived from spleen before treatment, contains an extra chromosome 18 and an extra structurally abnormal chromosome (M) derived mainly from a chromosome 3. Cell line SeF, derived from peripheral blood after chemotherapy, contains both of the abnormalities found in cell line SeD plus an apparently balanced translocation between a no. 7 and a no. 10 (arrows). Leukaemic cells circulating in the blood (post-treatment) had two chromosome complements: one (Blood 2) was like that of cell line SeD which had been derived 30 months before the blood samples were taken, the other (Blood 1) differed from that of SeD only in having an extra intact no. 3 instead of the no. 3-derived marker chromosome (M). The terminal dark band missing from the long arm of one no. 7 chromosome of Blood 2 (\*) was not found to be absent from any other cell; its significance is unknown.

from her leukaemic lymphocytes<sup>9</sup>. The modal number of chromosomes in line SeD was 48. This line's chromosome complement consisted of 46 apparently normal chromosomes, an extra no. 18 and an extra marker chromosome, M, which differs from any normal human chromosome as a result of structural rearrangement. The banding pattern of M suggests that it consists of the entire long arm, the centromere and a tiny segment of the short arm of a no. 3 to which a minute piece of some other chromosome has been translocated. The karyotype of cell line SeD can be described as 48, XX, +18, +M (Fig. 1). The extra chromosome 18 is apparently normal. Cell line SeF, a second leukaemic cell line, was derived from the patient's blood 2 yr after the establishment of line SeD and after chemotherapy with chlorambucil had been given. The leukaemic cell origin of this line was demonstrated by idiotype analysis in a similar manner to that for SeD<sup>9</sup>. As in SeD, a modal chromosome number of 48 was observed in SeF, but an additional chromosome abnormality was present, a translocation between the long arms of a no. 7 and a no. 10. The karyotype of line SeF can be described as 48, XX, -7, -10, +18, +M, +t(7p;10q) (Fig. 1). SeAl, one of the several lymphoblastoid cell lines from the same patient, derived from her non-leukaemic B cells<sup>9</sup>, had a normal 46, XX karyotype.

In the previous studies<sup>8</sup>, leukaemic B cells from patient Se matured to plasma cells *in vitro* in the presence of pokeweed

mitogen (PWM) and autologous T cells. Of the plasma cells identifiable with the anti-idiotypic serum to the monoclonal IgM serum band (IgM Se), 15% showed <sup>3</sup>H-thymidine incorporation by autoradiography. The majority of the dividing cells in the non-T population stained with anti-idiotypic antiserum and thus were of leukaemic origin<sup>8</sup>. To obtain metaphases from dividing leukaemic B cells, Se peripheral blood lymphocytes were stimulated with PWM for 3 d, after which T cells were separated from non-T cells by the sheep erythrocyte rosette method and the chromosome complements in each fraction analysed. All the metaphases obtained in the T-cell fraction had a normal karyotype, 46, XX. However, in the non-T-cell preparation, a bimodal distribution of chromosome numbers was observed, with the major mode having 48 chromosomes and the minor 46 chromosomes (Table 1). Of 50 G-banded cells, 24 had the complement 48, XX, +3, +18 and 5 had 48, XX, +18, +M (Fig. 1). A substantial number of the metaphases had a normal karyotype, which was probably from contaminating dividing normal cells. In addition, a normal karyotype was found in metaphases obtained directly from Se's freshly aspirated bone marrow cells as well as her peripheral blood lymphocytes stimulated with phytohaemagglutinin (PHA).

The chromosome abnormalities observed in the cell lines and the *in vitro* induced dividing peripheral blood leukaemic lymphocytes from patient Se are summarised in Fig. 1. Trisomy 3 and trisomy 18 seem to represent the earliest detected alterations in the patient's leukaemic clone. As the karyotype of the clone evolved, M replaced a chromosome 3 and, later, translocation between chromosomes 7 and 10 occurred. In chronic myelogenous leukaemia entering the blastic phase, sequential alterations of the karyotype of this type have been found to be superimposed on the Ph<sup>1</sup> translocation<sup>10</sup>. In CLL patient Se, correlation between the observed sequential alteration and altered clinical activity was not apparent.

Similar cytological studies were carried out on selected cell lines<sup>9</sup> derived from the peripheral blood of CLL patient Ei after she had been treated with chlorambucil and prednisone. An idiotype antiserum against the IgM Ei serum protein demonstrated that cell lines Ei 25, Ei 28 and Ei 81 were derived from her leukaemic lymphocytes<sup>9</sup>. All three leukaemic lines analysed had a modal chromosome number of 47, with the karyotype 47, XX, +12, and were, therefore, trisomic for what seems to be a normal chromosome 12. The lymphoblastoid lines Ei 151 and Ei 152 were shown by idiotype analysis to be derived from non-leukaemic B cells, and the karyotypes of these two non-leukaemic lines were normal (46, XX). The finding of trisomy 12 in all three independently derived leukaemic cell lines and its absence in the two normal lines suggest that the chromosome abnormality is not a result of changes produced by chemotherapy or EBV transformation and cell culture.

Simultaneous trisomy of chromosomes 3 and 18 has been described in the non-Hodgkin lymphomas<sup>5</sup>, although a structural rearrangement of chromosome 14 (14q+) is the most frequent abnormality<sup>1,2,5</sup>. A 14q+ chromosome has also been found in two patients with chronic T-cell leukaemia<sup>6</sup>, in two CLL patients with undetermined cell types<sup>5</sup> and in a patient with a B-cell CLL<sup>11</sup>. Trisomy 12 has been observed in at least two patients with hairy cell leukaemia<sup>12</sup>. In the present studies, the finding of trisomies 3 and 18 in patient Se and trisomy 12 in patient Ei suggest similar chromosome abnormalities in cases of CLL, malignant lymphoma and hairy cell leukaemia. None of

**Table 1** Chromosome numbers in pokeweed mitogen-stimulated peripheral blood mononuclear cells from patient Se after depletion of T cells

|           | Chromosome nos per cell |    |    |    |    |    | No. of cells examined |
|-----------|-------------------------|----|----|----|----|----|-----------------------|
|           | ≤ 44                    | 45 | 46 | 47 | 48 | 49 |                       |
| G-banding | 1                       | 6  | 11 | 2  | 29 | 1  | 50                    |
| Q-banding | 0                       | 0  | 6  | 4  | 20 | 0  | 30                    |
| Total     | 1                       | 6  | 17 | 6  | 49 | 1  | 80                    |

these chromosome abnormalities is known to be associated specifically with a particular type of lymphoproliferative disorder.

Thus, the leukaemic cells of both patients with CLL analysed in this study showed chromosome abnormalities. These were demonstrated in the *in vitro* stimulated cells from patient Se and the virus-transformed cell lines derived from both patients. The finding of normal karyotypes in the metaphases obtained from fresh bone marrow cells, PHA-stimulated cells and PWM-stimulated T cells in patient Se emphasises the difficulties inherent in cytogenetic studies of CLL. Without the idiotypic determinants as specific markers of the leukaemic cells, it would have been difficult to identify the leukaemic cells and demonstrate the associated chromosome abnormalities. The trisomy 3 and 18 in one patient and the trisomy 12 in the other are among the first well documented karyotypic abnormalities in chronic lymphocytic leukaemia of the common B-cell type. The fact that both patients studied showed abnormalities suggests that they occur at high frequency in cases of CLL but the exact incidence can be determined only when methods for identifying and inducing division of the leukaemic cells have been developed.

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## Inability of the C3a anaphylatoxin to promote cellular lysis

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Investigation of the biological effects of the cleavage product, C3a, derived from the third component of complement has generally focused on the effects it exerts as an anaphylatoxin<sup>1</sup>, that is, smooth muscle contraction<sup>2</sup> and histamine release from mast cells<sup>3,4</sup> and basophils<sup>5,6</sup>. Therefore, reports that C3a exerts cytotoxic effects on various cell types<sup>7-9</sup>, and exhibits concentration-dependent specificity for malignant cells<sup>9</sup>, excited great interest. The cytolytic activity mediated by macrophages which were activated *in vitro* may be attributed to their ability to generate C3a<sup>10</sup>. The far-reaching implications of such a cytolytic role for C3a led us to pursue these observations further. We report here that we have been unable to duplicate the results reported by Ferluga *et al.*<sup>9</sup>. On the contrary, we find that C3a actually reduces spontaneous release of label below normal background levels.

Human, porcine and rat C3a were purified according to established procedures<sup>11,12</sup>. Purity of the final products was assessed by polyacrylamide gel (pH 4.5) and cellulose acetate strip (pH 8.5) electrophoresis. All preparations were judged homogeneous based on the criteria of electrophoretic behaviour and a distinct amino acid composition. Functional activities of the anaphylatoxins were determined by the guinea pig ileal assay and from the ability to enhance vascular permeability in guinea pig skin<sup>1</sup>. Human C3a<sub>des Arg</sub> was prepared according to the procedure of Hugli *et al.*<sup>11</sup> except that the serum carboxypeptidase B inhibitor, 6-aminohexanoic acid, was omitted during the complement activation step.

Table 1 Effect of human C3a on murine target cells

| Expt 1                                         |                                                  | P-815 Cells        |                                                  |                    |
|------------------------------------------------|--------------------------------------------------|--------------------|--------------------------------------------------|--------------------|
| C3a concentration<br>( $\mu\text{g ml}^{-1}$ ) | <sup>51</sup> Cr release<br>(c.p.m. per culture) | % Specific release |                                                  |                    |
| 0                                              | 190 ± 29                                         | 0                  |                                                  |                    |
| 1                                              | 159 ± 19                                         | -5                 |                                                  |                    |
| 3                                              | 152 ± 22                                         | -6                 |                                                  |                    |
| 10                                             | 174 ± 5                                          | -2                 |                                                  |                    |
| 30                                             | 167 ± 3                                          | -3                 |                                                  |                    |
| 100                                            | 162 ± 18                                         | -4                 |                                                  |                    |
| Expt 2                                         |                                                  | P-815 cells        |                                                  | Spleen cells       |
| C3a concentration<br>( $\mu\text{g ml}^{-1}$ ) | <sup>51</sup> Cr release<br>(c.p.m. per culture) | % Specific release | <sup>51</sup> Cr release<br>(c.p.m. per culture) | % Specific release |
| 0                                              | 768 ± 46                                         | 0                  | 1,494 ± 59                                       | 0                  |
| 1                                              | 752 ± 86                                         | -1                 | 1,203 ± 101                                      | -30                |
| 3                                              | 738 ± 41                                         | -1                 | 1,112 ± 123                                      | -39                |
| 10                                             | 715 ± 19                                         | -2                 | 1,051 ± 54                                       | -45                |
| 30                                             | 661 ± 57                                         | -5                 | 1,007 ± 87                                       | -49                |
| 100                                            | 636 ± 17                                         | -6                 | 727 ± 29                                         | -78                |

In expt 1 a total of  $5 \times 10^6$  P-815 cells were labelled with <sup>51</sup>Cr-labelled sodium chromate in 1 ml medium and incubated at 37 °C for 1 h. Total release was  $868 \pm 0$  c.p.m. <sup>51</sup>Cr release was assayed after 6 h of incubation. Each culture contained  $3.5 \times 10^4$  target cells + the concentration of C3a indicated. In expt 2,  $5 \times 10^6$  P-815 or spleen cells were labelled with <sup>51</sup>Cr-labelled sodium chromate in 0.3 ml medium and incubated at 37 °C for 1 h. Total release was  $3,131 \pm 89$  c.p.m. for P-815 cells and  $2,480 \pm 57$  c.p.m. for spleen cells. The per cent specific release of label from target cells attributable to the action of C3a was calculated according to the formula:

$$\frac{(\text{Experimental release} - \text{spontaneous release})}{(\text{Total release} - \text{spontaneous release})} \times 100$$

Total release was evaluated by adding a drop of 10% SDS to cultures containing target cells only. Data are presented as the arithmetic mean of triplicate cultures ± s.e.

Dissociated suspensions of murine spleen cells were prepared and cultured in RPMI-1640 based medium as detailed elsewhere<sup>13</sup>. This medium was supplemented with bovine serum albumin (BSA) or heat-inactivated fetal calf serum (FCS) as indicated. FCS was tested for and found deficient in carboxypeptidase B activity. The H-Z<sup>d</sup> mastocytoma P-815 and the H-2<sup>b</sup> lymphoma EL-4 were maintained *in vivo* in DBA/2 and C57BL/6J mice, respectively. Target cells were labelled according to several different protocols. For the first protocol,  $5 \times 10^6$  viable target cells were suspended in 1 ml of medium together with 100  $\mu\text{Ci}$  of <sup>51</sup>Cr-sodium chromate, and incubated for 1 h at 37 °C. Target cells were washed four times and resuspended at a density of  $3.5 \times 10^5$  per ml in medium containing 0.1% crystallised BSA. In other experiments the same labelling protocol was followed except that the total volume of incubate was 0.3 ml. The use of this latter protocol resulted in a higher degree of labelling per cell. Several other labelling techniques were tested to optimise the amount of label taken up per



**Table 2** Effect of C3a from different species on P-815 target cells

| C3a<br>( $\mu\text{g ml}^{-1}$ ) | Human C3a                                          |                       | Rat C3a                                            |                       | Porcine C3a                                        |                       | Human C3a <sub>des Arg</sub>                       |                       |
|----------------------------------|----------------------------------------------------|-----------------------|----------------------------------------------------|-----------------------|----------------------------------------------------|-----------------------|----------------------------------------------------|-----------------------|
|                                  | <sup>3</sup> H-TdR release<br>(c.p.m. per culture) | %<br>Specific release | <sup>3</sup> H-TdR release<br>(c.p.m. per culture) | %<br>Specific release | <sup>3</sup> H-TdR release<br>(c.p.m. per culture) | %<br>Specific release | <sup>3</sup> H-TdR release<br>(c.p.m. per culture) | %<br>Specific release |
| 0                                | 6,513 $\pm$ 375                                    | 0                     | 6,513 $\pm$ 375                                    | 0                     | 6,513 $\pm$ 375                                    | 0                     | 380 $\pm$ 100                                      | 0                     |
| 1                                | 4,773 $\pm$ 418                                    | -3.3                  | 5,987 $\pm$ 431                                    | -1.0                  | 4,998 $\pm$ 173                                    | -2.9                  | 335 $\pm$ 34                                       | -0.2                  |
| 3                                | 4,091 $\pm$ 211                                    | -4.6                  | 6,024 $\pm$ 190                                    | -0.9                  | 3,861 $\pm$ 135                                    | -5.0                  | 345 $\pm$ 15                                       | -0.2                  |
| 10                               | 3,568 $\pm$ 108                                    | -5.6                  | 5,373 $\pm$ 331                                    | -2.0                  | 4,974 $\pm$ 287                                    | -2.9                  | 298 $\pm$ 11                                       | -0.4                  |
| 30                               | 2,592 $\pm$ 27                                     | -7.4                  | 4,604 $\pm$ 364                                    | -3.6                  | 4,005 $\pm$ 109                                    | -4.7                  | 269 $\pm$ 26                                       | -0.5                  |
| 100                              | 2,820 $\pm$ 42                                     | -7.0                  | 3,469 $\pm$ 188                                    | -5.8                  | 3,610 $\pm$ 238                                    | -5.5                  | 231 $\pm$ 13                                       | -0.7                  |

<sup>3</sup>H-TdR release was assayed after 6 h of incubation. Each culture contained  $5 \times 10^4$  target cells plus the concentration of C3a indicated. Total release was  $59,427 \pm 4,386$  c.p.m. per culture. Data are presented as the arithmetic mean of five replicate cultures  $\pm$  standard error. For the human C3a<sub>des Arg</sub> experiment <sup>3</sup>H-TdR release was also assayed after 6 h of incubation and each culture contained  $5 \times 10^4$  target cells. The concentration of C3a<sub>des Arg</sub> is indicated and total release was  $23,010 \pm 530$  c.p.m. per culture. Release in the cultures with  $100 \mu\text{g ml}^{-1}$  of intact C3a instead of C3a<sub>des Arg</sub> was  $195 \pm 12$  c.p.m. per culture (-0.8% specific release).

cell. The most satisfactory protocol on this basis, was that described by Bloom *et al.*<sup>14</sup>. Briefly, target cells were incubated in the same RPMI-1640 based medium used previously with the exception that BSA was omitted and 10% heat-inactivated FCS as well as  $5 \times 10^{-5}$  M 2-mercaptoethanol were added.  $10^7$  target cells were incubated in 60-mm Petri dishes with  $5 \mu\text{Ci ml}^{-1}$  of <sup>3</sup>H-TdR in a volume of 10 ml. Cultures were incubated for 18–24 h, washed four times, resuspended to  $10^6$  cells per ml in serum-free medium and used directly.

<sup>51</sup>Cr-labelled target cells were assayed according to the procedure of Ferluga *et al.*<sup>9</sup>. Briefly,  $3.5 \times 10^4$  target cells in a volume of 0.1 ml medium were combined with various concentrations of C3a (in 20  $\mu\text{l}$  of gelatin-saline) in  $12 \times 75$ -mm round bottomed tubes and incubated in an atmosphere of 5% CO<sub>2</sub> at 37°C for the time indicated. At that point, 1 ml of 5% FCS-containing medium was added per tube, samples were mixed gently, centrifuged, and 0.5 ml of the supernatant was removed and counted in a Searle 1195 automatic gamma counter. Target cells labelled with <sup>3</sup>H-TdR were assayed as follows:  $5 \times 10^4$  target cells in a volume of 50  $\mu\text{l}$  were added to microculture wells (Falcon Plastics no. 3040, 3041) together with a  $2 \times$  concentration of human C3a in a volume of 50  $\mu\text{l}$  of gelatin-saline. Cells were incubated for 6 h unless otherwise stated, at which point the assay was terminated by adding 100  $\mu\text{l}$  of medium containing 10% FCS to each well. The microculture plate was then centrifuged and 100  $\mu\text{l}$  per culture was harvested into scintillation vials containing 3 ml of Aquasol. Samples were counted in a Beckman LS-230 liquid scintillation counter.

The data presented in Table 1 are results from experiments in which variable concentrations of human C3a were incubated with two different types of radiolabelled target cells. Significant release of label from the target cells, an event that would indicate lysis initiated by C3a, failed to occur. Although these experiments were performed repeatedly and the methodology specified by Ferluga *et al.*<sup>9</sup> was adhered to fastidiously, lysis was never observed. This was true of <sup>51</sup>Cr-labelled P-815 target cells, the cell type reported to be the most readily lysed, as well as EL-4 and normal murine splenic lymphocytes. The susceptibility of these target cells to lysis, as judged by their release of the <sup>51</sup>Cr label, was previously established by incubating the cells with allogeneic cytotoxic T lymphocytes which had been specifically educated *in vivo*. The range of C3a concentrations tested was precisely that which has been previously reported to produce lytic activity against labelled target cells<sup>9</sup>. The assays presented in Table 1 were performed after 6 h of incubation, as specified by Ferluga *et al.*<sup>9</sup>. However, in other experiments (data not shown) the kinetic profile (between 2 and 24 h of culture) was examined. The results of these experiments demonstrated that lysis could not be induced regardless of the duration of the

incubation. Viability studies showed no differences in cell number or in per cent viability between control and C3a-incubated cultures (data not shown).

As our next approach, highly purified C3a preparations of human, rat, and porcine origin were studied to determine whether the inability of human C3a preparations to lyse target tumour cells might be simply a species limitation. The results of experiments using <sup>3</sup>H-TdR-labelled P-815 mastocytoma target cells are presented in Table 2. Here again, the actual counts released per culture, as well as the per cent specific release, are presented as a function of the concentration of C3a used in culture. Once more it is apparent that there is no lysis induced by C3a in excess of the background spontaneous release regardless of its species derivation, over the entire range of concentrations examined in Table 1. Although guinea pig C3a was used in the studies of Ferluga *et al.*<sup>9</sup>, the use of C3a from a variety of species (Table 2) makes it unlikely that cytolysis is peculiar to the guinea pig molecule especially since the target cells were xenogeneic.

**Table 3** Effect of polyamines on spontaneous lysis of murine tumour cells

| Concentration<br>( $\mu\text{g ml}^{-1}$ ) | Human C3a                                          |                       | Spermidine                                       |                       |
|--------------------------------------------|----------------------------------------------------|-----------------------|--------------------------------------------------|-----------------------|
|                                            | <sup>3</sup> H-TdR release<br>(c.p.m. per culture) | %<br>Specific release | <sup>51</sup> Cr release<br>(c.p.m. per culture) | %<br>Specific release |
| 0                                          | 5,826 $\pm$ 440                                    | 0                     | 5,826 $\pm$ 440                                  | 0                     |
| 1                                          | 3,524 $\pm$ 90                                     | -4.5                  | 3,599 $\pm$ 173                                  | -4.3                  |
| 3                                          | 3,702 $\pm$ 124                                    | -4.1                  | 2,829 $\pm$ 223                                  | -5.8                  |
| 10                                         | 3,300 $\pm$ 34                                     | -4.9                  | 1,735 $\pm$ 128                                  | -7.9                  |
| 100                                        | 3,143 $\pm$ 97                                     | -5.2                  | 589 $\pm$ 34                                     | -10.2                 |

<sup>3</sup>H-TdR release was assayed after 6 h of incubation. Each culture contained  $5 \times 10^4$  P-815 target cells plus the concentration of C3a or spermidine indicated. Total release was  $57,328 \pm 5,553$  c.p.m. per culture in these experiments.

Moreover, it appears that if any activity can be ascribed to the presence of C3a in culture, that activity would be a diminution of the spontaneous release of radioactive label from target cells. The fact that biologically inactive C3a<sub>des Arg</sub> exhibits the same effect as intact C3a on spontaneous release argues that this phenomenon is not an anaphylatoxin-specific effect.

In other studies, the effect of C3a on isologous target cells was studied. Human lymphoblasts, generated from PBL (activated by staphylococcal protein A under optimal mitogenic conditions for 6 d) were incubated with concentrations of human C3a

ranging from 1 to 200  $\mu\text{g ml}^{-1}$ . The results of these experiments demonstrated that purified human C3a was incapable of mediating the release of label from human lymphoblastoid target cells.

Theoretically C3a should be able to interact with cells either by high or low affinity receptor-ligand binding, or by low affinity nonspecific interactions (such as salt bridging and hydrogen bonding). In a recent paper, Glovsky and colleagues<sup>6</sup> studied the binding of purified human C3a to human eosinophils, neutrophils, basophils, monocytes and lymphocytes. Of particular interest was their observation that virtually no detectable binding of human C3a to peripheral blood lymphocytes occurred. In the light of these findings, it is difficult to comprehend the rational basis for specific lysis of human lymphoblasts by C3a observed by Ferluga and coworkers<sup>9</sup>. One would have to postulate a nonspecific or low affinity mechanism to account for such findings since high affinity binding of C3a to a specific cellular receptor is apparently ruled out.

On the contrary, diminution of spontaneous cytolysis by C3a was conceptualised as a nonspecific effect of polycations. To evaluate this possibility salmon spermidine, a polyamine of comparable size to C3a, was evaluated for its influence on the spontaneous lysis of labelled target cells. Table 3 demonstrates that increasing the concentration of salmon spermidine does in fact produce a diminution in the spontaneous release of cellular label. The effect of spermidine in our case is not likely to be due to inhibitory metabolites produced by polyamine oxidase contained in FCS acting on spermidine as a substrate<sup>15</sup>. It has been demonstrated that inhibitory effects attributable to such metabolites are reversed by washing, and in the present studies cells were washed four times before addition of spermidine in serum-free medium. Moreover, the same effects of polycations were observed when target cells were labelled in the absence of serum. The decrease in spontaneous release is interpreted as indicating that polyamines, similar in size and charge to C3a, are capable of exerting either direct or indirect nonspecific stabilising effects on the cell. This same conclusion has been independently corroborated by experiments in which human C3a was incubated with human peripheral blood neutrophils. Under those circumstances, the spontaneous release of lactate dehydrogenase (LDH) decreased concordantly with increasing concentrations of C3a (D. E. Chenoweth, personal communication).

We thus find no evidence to support the contention that C3a plays a significant cytolytic role in the host defense mechanisms against neoplasia.

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## Prostaglandin synthesis in different phases of phagocytosis in lung macrophages

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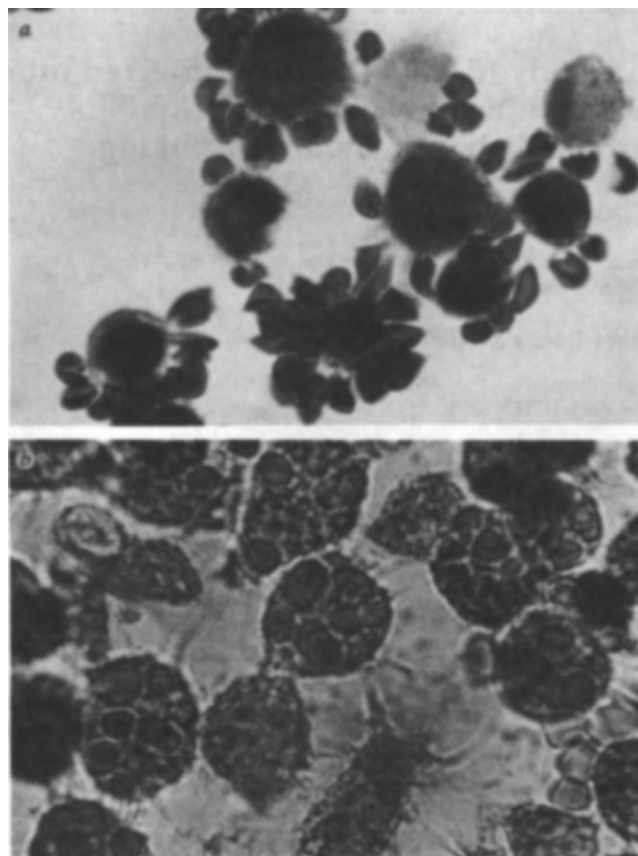
Macrophages have a central role in bodily defence and inflammatory responses. Prostaglandins (PGs), mediators of inflammation<sup>1</sup>, are secreted by macrophages during phagocytosis. PGE-like activity was first demonstrated in macrophage-rich peritoneal exudate cell preparations from guinea pigs<sup>2-3</sup>; later, other kinds of PGs were also found to be released by mouse peritoneal macrophages in response to inflammatory stimuli<sup>4-6</sup>. We have shown that rabbit alveolar macrophages also produce various PGs in response to phagocytic stimuli like zymosan and heat-killed bacteria<sup>7,8</sup>; however, it was not known precisely which cellular event is associated with PG production. Traditionally, the phagocytic process is considered in three stages: (1) attachment of the particle to the cell membrane; (2) interiorisation (phagocytosis); and (3) fusion of the phagocytic vesicle with intracellular lysosomes (digestion). We show here that PG secretion in response to phagocytic stimuli by macrophages is independent of the first stage, but dependent on engulfment of the particle. Moreover, PG production does not appear to be associated with the process of engulfment *per se*, but with some event following internalisation.

To assess the release of PGs, we prelabelled the culture rabbit alveolar cells (>90% macrophages) with <sup>14</sup>C-arachidonic acid (Amersham Searle), as previously described<sup>7</sup>. Analysis of the cell lipids showed that approximately 40-50% of the radioactive arachidonic acid was incorporated into the cell lipids after 1 h of incubation. Most of the radioactivity (68%) resided in phospholipid (41±2% phosphatidyl choline, 17±3% phosphatidyl ethanolamine, and 10±2% phosphatidyl serine and phosphatidyl inositol) (*n* = 10). A small amount (9±2%) was also incorporated into the neutral lipids; only 1-2% of the radioactivity in the cells remained in free fatty acid form. The pattern of incorporation was similar to our previous observation<sup>7</sup>.

It has been also shown that concanavalin A (Con A)-treated red blood cells (RBCs) attach to the macrophages, but are not engulfed<sup>9</sup>. We took advantage of this model to study the attachment phase of phagocytosis. As shown in Fig. 1a, the Con A-treated RBCs bound to the alveolar macrophages to form rosettes, but none was engulfed at the end of the incubation period (3 h). The PG production of these macrophages was no different from that of the unstimulated group (Fig. 2a). However, when anti-rabbit RBC antiserum was added to the culture media together with rabbit RBCs, the latter were rapidly interiorised (Fig. 1b). Accordingly, the PG secretion showed a 60% increase compared with the control group (Fig. 2a). Neither RBCs nor antiserum alone caused any increase of PG synthesis by macrophages. Thus, the PG release by macrophages during RBC phagocytosis is not dependent on attachment of the RBCs to the macrophage membrane, but depends on the engulfment of the RBCs by these phagocytic cells. This result differs from observations of Goldstein *et al.*<sup>10</sup> in other phagocytic cells such as granulocytes. These cells, once rendered incapable of ingesting particles by treatment with cytochalasin B, generated comparable amounts of thromboxane B<sub>2</sub> to untreated, normal cells.

PG release following RBC phagocytosis by rabbit alveolar macrophages was predominantly PGE<sub>2</sub> (38±4% of total PG released), although PGD<sub>2</sub> (14±2%), PGF<sub>2</sub>α (25±2%), and





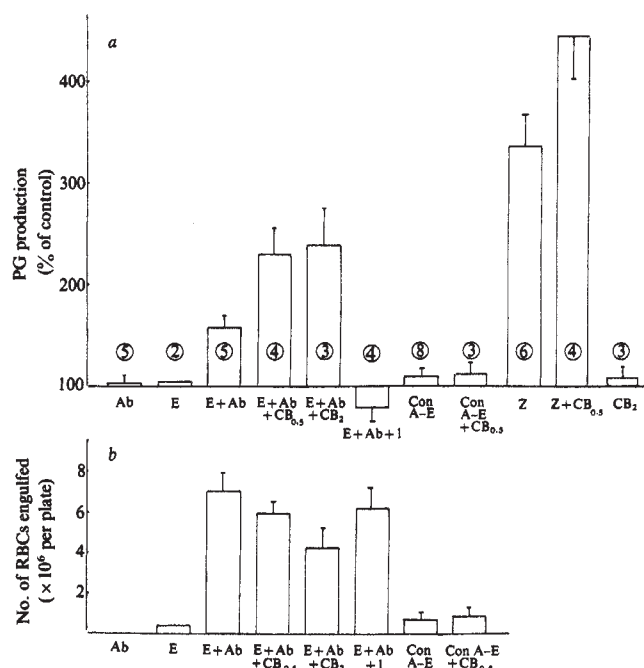
**Fig. 1** *a*, Attachment of Con A-treated RBCs to cultured alveolar macrophages. RBCs were collected, washed twice with phosphate-buffered saline (PBS) (pH 7.4), resuspended in PBS containing  $0.7 \mu\text{g ml}^{-1}$  Concanavalin A (Sigma), and incubated at  $37^\circ\text{C}$  for 30 min. The RBCs were then washed twice with PBS and  $1 \times 10^7$  RBCs were added to cultured alveolar macrophages ( $5 \times 10^6$  cells  $\text{ml}^{-1}$  on each plate). At the end of experimental period, the cells were fixed *in situ* with 10% formalin and stained with haematoxylin and eosin (H and E). *b*, Engulfment of RBCs by cultured alveolar macrophages. The anti-rabbit RBC antiserum was prepared from mice by a modified method of Rabinovitch<sup>11</sup>. A group of 10 mice were injected twice intravenously with 0.2 ml of 10% rabbit RBCs suspended in PBS. The interval between injections was 30 d, and the mouse serum was collected 6 d after the second injection. Fresh rabbit RBCs were collected and washed twice with PBS and  $1 \times 10^7$  cells were added to alveolar macrophages ( $5 \times 10^6$  cells per plate) in the presence of anti-rabbit RBC antiserum. The cells were fixed *in situ* with 10% formalin at the end of experimental period and stained with H and E stain.

6-keto  $\text{PGF}_1\alpha$  ( $22 \pm 3\%$ ) ( $n = 5$ ) were also detected in the medium, with a profile very similar to that of zymosan-stimulated alveolar macrophages<sup>7,8</sup>.

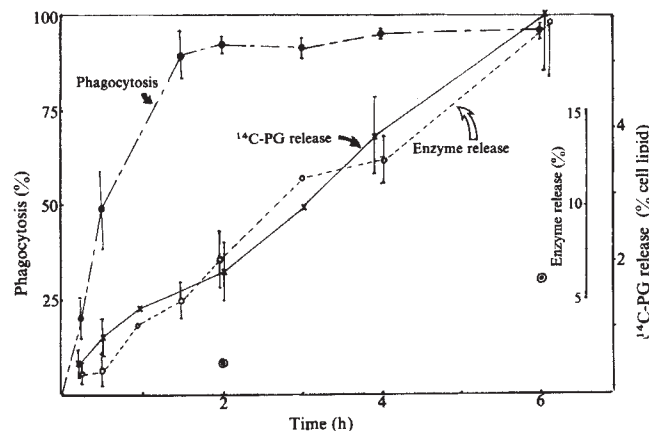
Our previous work also showed that cytochalasin B, an inhibitor of phagocytosis, when given to zymosan-treated macrophages, increased PG production instead of inhibiting it<sup>7</sup>. In the absence of zymosan, however, cytochalasin B exhibited no effect on PG production<sup>7</sup>. One possible explanation for this paradoxical phenomenon is that PGs are produced during particle-membrane interaction, and that by inhibiting engulfment of the particles, cytochalasin B prolongs the attachment phase, that is, the particle-membrane interaction, thus enhancing PG production. If this hypothesis obtained, we should be able to see a stimulatory effect of cytochalasin B on PG production, when Con A-treated RBCs were used as a stimulus for particle attachment. As shown in Fig. 2*a*, this hypothesis is clearly false. There was no increase of PG secretion by macrophages when cytochalasin B was added to Con A-treated RBCs. However, when RBCs were engulfed in the presence of anti-

RBC antiserum, a facilitatory effect of cytochalasin B on PG secretion was clearly demonstrated. This increment was demonstrable at both doses that inhibited phagocytosis ( $2 \mu\text{g ml}^{-1}$  and below  $0.5 \mu\text{g ml}^{-1}$ ). Therefore, we have shown that: (1) the PG stimulating effect of cytochalasin B was not a result of inhibition of phagocytosis or prolongation of particle-membrane interaction; and (2) the stimulatory effect of cytochalasin B was dependent on the interiorisation of the phagocytosed particles.

Finally, we investigated the relationship between particle engulfment and PG secretion. Zymosan was used instead of



**Fig. 2** *a*,  $^{14}\text{C}$ -PG production by rabbit alveolar macrophages in response to different stimuli. Rabbit alveolar macrophages were collected as previously described<sup>7</sup> ( $5 \times 10^6$  cells  $\text{ml}^{-1}$  on each plate). After overnight incubation, the cells were changed to serum-free Dulbecco's modified Eagle's medium (DMEM).  $^{14}\text{C}$ -arachidonic acid (specific activity  $55 \text{ mCi mmol}^{-1}$ , Amersham Searle) was added to the cultures as the Na salt at a concentration of  $1 \times 10^6 \text{ c.p.m. ml}^{-1}$ . The cultures were incubated for 1 h to label the cell lipids. Cells were washed twice with DMEM, different stimuli were added to the cells and they were incubated for 2.5 h in DMEM containing penicillin ( $100 \mu\text{g ml}^{-1}$ ), and streptomycin ( $100 \mu\text{g ml}^{-1}$ ). At the end of the experimental period, the medium was collected, acidified to pH 3.5 with formic acid, extracted twice with an equal volume of ethyl acetate, dried and applied to silica gel G plates. The solvent system used for separation of PGs was the organic phase from ethyl acetate:water:iso-octane:acetic acid, 110:100:50:20 (ref. 12). Zones corresponding to various PG standards (provided by Dr John Pike, Upjohn, Kalamazoo) were scraped and counted in a liquid scintillation counter. The total PG release (sum of 6-keto  $\text{PGF}_1\alpha$  +  $\text{PGF}_2\alpha$  +  $\text{PGE}_2$  +  $\text{PGD}_2$ ) was calculated and expressed as the percentage of the control value (unstimulated cells). E, PBS-washed rabbit erythrocytes ( $1 \times 10^7$  cells per plate); Ab, anti-rabbit RBC antiserum ( $25 \mu\text{l ml}^{-1}$ ); CB<sub>0.5</sub>, cytochalasin B ( $0.5 \mu\text{g ml}^{-1}$ ); CB<sub>2</sub>, cytochalasin B ( $2 \mu\text{g ml}^{-1}$ ); Con A-E, ConA ( $0.7 \mu\text{g ml}^{-1}$ ) treated RBCs ( $1 \times 10^7$  cells per plate); Z, zymosan ( $1 \text{ mg ml}^{-1}$ ); I, indomethacin ( $2 \mu\text{g ml}^{-1}$ ). The encircled numbers indicate the number of experiments performed (mean  $\pm$  SEM indicated). *b*, Intracellular haemoglobin (Hb) content of macrophages. At the end of the experimental period (2.5 h), the medium was removed and  $\text{NH}_4\text{Cl}$  in saline ( $8.3 \text{ mg ml}^{-1}$ ) was added to cells to lyse the extracellular RBCs. The medium was again removed, the macrophages were scraped off to a test tube, and Hb content was determined by the cyanmethaemoglobin method<sup>13</sup>. A series of standards of different concentrations of RBCs were used and the Hb content in cells were expressed as number of RBCs in each dish of macrophages ( $5 \times 10^6$  cells  $\text{ml}^{-1}$  on each plate). (Mean  $\pm$  s.e.m. indicated.)



**Fig. 3** Time course of phagocytosis of zymosan, lysosomal enzyme release, and PG production by alveolar macrophages. Zymosan was added at time zero, and media were collected at different time intervals for analysis of acid phosphatase activity and PG production. Cells were fixed with 75% alcohol and stained with PAS reaction and the percentage of cells containing two or more zymosan particles (magenta colour by PAS stain) was recorded. The total PG production was determined by radiochemical techniques as described in Fig. 2a, and is expressed as percentage of total cell lipid count. The acid phosphatase activity was determined on both medium and cells by a modification of the procedure of Leaback<sup>14</sup>. To determine the intracellular enzyme content, the cells were scraped off the plates into 0.5 ml of 0.1% Triton X-100 in water using a rubber policeman. Aliquots of the cell lysates were diluted 50 times with 0.2 M sodium acetate buffer (pH 5). The media were diluted five times with the same buffer. Thirty  $\mu$ l of the diluted medium or cell lysate were added to 70  $\mu$ l of 4-methylumbelliferyl phosphate (0.05 mg ml<sup>-1</sup>) in acetate buffer and incubated at 37°C for 15 min. The enzyme reaction was terminated by adding 1.9 ml of 0.02 M phosphate buffer (pH 10.7). The fluorescence was measured in a fluorimeter using a Corning 5860 primary filter and Corning 3387 and 5543 secondary filters. The release of enzyme is expressed as percentage of intracellular acid phosphatase released into the medium (mean  $\pm$  s.e.m.,  $n = 3$ ).  $\times$ ; <sup>14</sup>C-PG production;  $\circ$ ; percentage of intracellular acid phosphatase released into the medium after zymosan stimulation;  $\odot$ ; percentage of intracellular acid phosphatase released into the medium by unstimulated cells;  $\bullet$ ; percentage of cells phagocytosing zymosan particles.

RBCs, due to its potent stimulatory effect. As shown in Fig. 3, PG release did not temporally follow the particle engulfment, but exhibited a time lag. Its time course, however, followed closely that of the release of lysosomal enzymes. Furthermore, we have previously shown that cytochalasin B, which increased zymosan-induced release of PGs, also increased that of lysosomal enzymes<sup>7</sup>. Hence, it seems that the release of PGs by alveolar macrophages during phagocytosis is not associated with either the attachment or engulfment phases, but it is concomitant with a much later event, which takes place after the particles have been internalised.

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## Antibody to Thy-1 antigen injected into rat hypothalamus selectively inhibits carbamyl choline induced drinking

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The Thy-1 differentiation antigen on the cell surfaces of mouse and rat thymocytes, lymphocytes<sup>1–4</sup>, epidermis<sup>5</sup>, developing skeletal muscle<sup>6</sup>, and brain<sup>1,3,4</sup> has not yet been assigned a function. In rat and mouse brain, Thy-1 reaches adult levels at about 3 weeks of age from minimal or variably low levels at birth<sup>3,7–9</sup>. *In vitro* studies of fetal or newborn cerebellar neurons have shown that granule cells and some large multipolar cells become Thy-1 positive during culture<sup>10,11</sup>. All dorsal root ganglion neurones in culture express Thy-1<sup>11,12</sup>. The antigen appears to be enriched in synaptosomal fractions<sup>13,14</sup> but Thy-1 has recently been demonstrated on the surface and dendrites of microdissected neurones of adult rat brain<sup>15</sup>. In the brain Thy-1 has been thought to be a neuronal antigen, but some cerebellar astrocytes become Thy-1 positive after prolonged culture<sup>16</sup>. The transient or developed occurrence of the Thy-1 glycoprotein on selected cell surfaces would suggest that it mediates an event associated either with differentiation or with a differentiated function. The recent report by Dulbecco *et al.* that monoclonal hybridoma antibody against Thy-1.1 (aThy-1.1) blocks a differentiation event in culture of a rat mammary carcinoma cell line<sup>17</sup> is, the first demonstration of a functional link between the molecule and a specific developmental process. We consider brain Thy-1 to be a differentiated adult expression, however, and we report here that injecting antibody to Thy-1 antigen into the rat hypothalamus caused selective inhibition of carbamyl choline induced drinking.

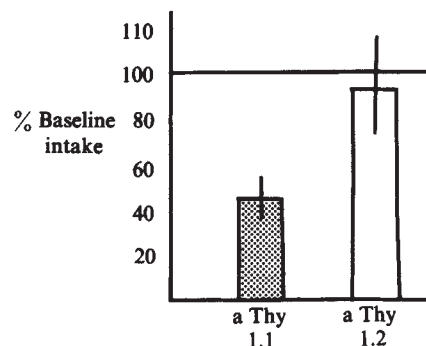
Direct chemical stimulation of the perifornical hypothalamus with the  $\alpha$ -adrenergic agonist noradrenaline (NA) elicits food intake in satiated rats. Stimulation of the cholinergic system at the same site with the acetylcholine analogue carbamyl choline (CC) elicits a sustained drinking response in water-replete rats<sup>18–21</sup>. If the effect of focal antibody treatment is a result of reaction with antigens characteristic of differentiated neuronal function, then a selective potentiation or inhibition of the eating or drinking response to an exogenous transmitter analogue might be anticipated.

Monospecific antibody against Thy-1 antigens (aThy-1) are produced by hybridising mouse myeloma and immune spleen cells, cloning the hybridoma and culturing as a tumour *in vivo*. Hybridoma aThy-1.1 (T32B11) mouse serum, cytotoxic (complement dependent) for AKR mouse thymocytes at a dilution of  $1 \times 10^{-5}$ , and a monoclonal aThy-1.2 (F7D5), cytotoxic for C57BL mouse thymocytes at a dilution of  $1 \times 10^{-6}$  (ref. 22), were kindly supplied by Dr E. A. Clark. Both antisera were included in the study by Dulbecco *et al.*<sup>17</sup> of the effect on the differentiation of mammary cell cultures. Both were tested in our laboratory by indirect immunofluorescence for specific binding to mouse and rat thymocytes. aThy-1.2 bound to C3H cells but not to AKR or rat cells; aThy-1.1 bound to both AKR and rat cells but not to C3H cells, confirming the designated specificity of these antibody preparations. The antibody is not thought to react with carbohydrate determinants since the brain Thy-1 carbohydrate has a different composition from that of the thymocyte Thy-1<sup>23–25</sup>.



Twenty-four male Charles River CD rats were implanted with chronic unilateral cannulae aimed at the left perifornical hypothalamus. Following surgery and a 2-week recovery period, the animals were divided into two groups to be tested with NA (6  $\mu$ g) and physiological saline (PS), or with CC (0.8  $\mu$ g) and PS to determine baseline eating and drinking responses to chemical stimulation. Drug doses were chosen to produce a vigorous behavioural response. Tests were performed at 4-day intervals in a counterbalanced schedule to detect any sequence effects. Half of the animals received the drug (NA or CC) and half received PS on the first day. In the next test the animals were injected with the other substance. All tests were performed at 4 pm, 2 hours before the onset of the dark period of the light-dark cycle. To initiate behavioural arousal and ensure maximal satiation at the start of the test, each rat was given fresh food and water 30 min before the test period. Immediately after this presatiation period, the animals were removed from their home cages, injected with 1  $\mu$ l of the test solution (NA, CC or PS) and returned to the cage which contained a fresh amount of premeasured food and water. After 1 hour the remaining food was removed from the cage and, with the spillage, weighed to the nearest 0.1 g to determine food intake. Water intake was read to the nearest 1 ml.

The effect of aThy-1.1 on drug-induced behaviours was determined using the same counterbalancing procedure. Tests were performed at weekly intervals. On test days half of the animals in each group received two 1- $\mu$ l intrahypothalamic injections of aThy-1.1 at 10 and 10:30 am, 6 hours before the



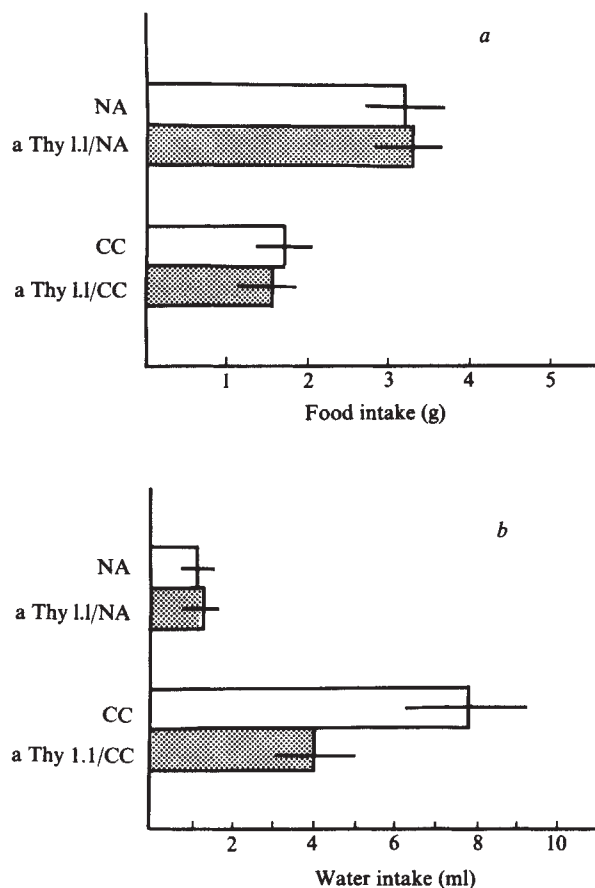
**Fig. 2** Comparison of the effects of intrahypothalamic monoclonal antibodies T32B11 (aThy-1.1) and F7D5 (aThy-1.2) on carbamyl choline (CC) stimulation. Data are expressed as percentage of water intake by carbamyl choline (0.8  $\mu$ g in 1  $\mu$ l)-stimulated animals following treatment with aThy-1.1 or treatment with aThy-1.2 (serum diluted 1/5) 6 h before injection of drug into the perifornical hypothalamus. ( $n = 12$ .) Error bars show  $\pm$ s.e.m.

chemical stimulation and consumption test period. For injection into the brain site aThy-1.1 serum was diluted fivefold to 14  $\mu$ g protein per  $\mu$ l since protein concentrations of 25–35  $\mu$ g  $\mu$ l<sup>-1</sup> at this site sometimes gave nonspecific effects on consummatory behaviours.

Figure 1 shows that NA elicited excessive eating while CC elicited excessive drinking, confirming the work of other investigators on the adrenergic and cholinergic regulation of appetitive behaviour at this site<sup>18–21</sup>. Drinking after NA injection and eating after CC injection did not differ significantly from consumption in saline control conditions. Following treatment with aThy-1.1 antibody the drinking response to CC was significantly reduced ( $P < 0.02$ ), whereas neither the drinking nor the eating responses in any other treatment conditions were significantly altered. Repeated baselines with CC indicated complete recovery of the drinking response by 3 days.

These animals were also tested with aThy-1.2, specific for the alloantigenic variant not found in the rat. This was done to control for the possibility that the selective reduction of CC induced drinking was a nonspecific effect, either of some mouse serum component or a hybridoma secretion other than antibody. aThy-1.2 was used at the same protein concentration as aThy-1.1, but only CC-stimulated drinking was tested since that was the only behaviour affected by aThy-1.1. Figure 2 shows the effects of aThy-1.1 and aThy-1.2 on CC-stimulated drinking. Treatment with aThy-1.1 reduced CC-stimulated drinking by >50%, whereas the reduction of mean water intake observed after treatment with aThy-1.2 was not significantly different from the CC baseline. Although aThy-1.1 and aThy-1.2 were allotype-specific, the effect of the high serum concentration of the initial experiment might have been due to some other antibody or substance in the aThy-1.1 serum. The animals were tested, therefore, in groups of eight at aThy-1.1 dilutions of 100, 500, 5,000 and 20,000, alternating with repeat baseline determinations at weekly intervals. Six animals not consistently responding to CC stimulation in three baseline determinations were eliminated from this study. aThy-1.1 diluted 100- and 500-fold significantly reduced the drinking response to CC stimulation ( $P < 0.01$ ). It is probable, however, that dilutions higher than 500 will show a significant effect in larger groups. Because of the activity of aThy-1.1 at 500 dilutions, equivalent to less than 1 pmol of immunoglobulin, we concluded that the effect was Thy-1 specific.

These experiments suggest that the Thy-1 antigen in the rat hypothalamus is distributed in such a way that its reaction with antibody results in a blockade of cholinergic function. The distribution of Thy-1 by structures and brain regions<sup>1,9,15,26</sup> is not inconsistent with a link between Thy-1 and cholinergic function. However, the detection of Thy-1 over the entire surface of



**Fig. 1** Drug-induced eating (a) and drinking (b) modified by intrahypothalamic administration of monoclonal antibody T32B11 to Thy-1.1 antigen (aThy-1.1). Open bars are predetermined drug baseline consumption data. Stippled bars are drug-stimulated consumption data following aThy-1.1 treatment (serum diluted 1/5) administered 6 h before injection of drug into the perifornical hypothalamus. Noradrenaline (NA), 6  $\mu$ g, and carbamyl choline (CC), 0.8  $\mu$ g, were administered in 1  $\mu$ l. ( $n = 12$ .) Error bars show  $\pm$ s.e.m.

dorsal root ganglion neurones, which are thought to have only dendritic innervation<sup>11,12</sup>, suggests that Thy-1 localisation is not exclusively associated with synapses. Others report that brain Thy-1 is predominantly in the synaptosomal fraction<sup>13,14</sup>. Presynaptic Thy-1 has been demonstrated on aminergic synaptosomes by complement-mediated cytolytic release of transmitters<sup>27</sup>, the antigen might still be associated with cholinergic modulation of normal release mechanisms.

Our experiments indicate no association of rat brain Thy-1 with  $\alpha$ -adrenergic function, at least none which can be blocked by an antibody to Thy-1.1. In an earlier study using the same behavioural test we found complete suppression of NA-induced eating by two different rabbit antisera against a synaptic membrane fraction (aSM). No effect on CC-induced drinking was detectable with aSM. Conversely, a rabbit antiganglioside serum (aGS) greatly reduced the drinking response to CC without affecting NA-induced eating<sup>28</sup>. That study represents an important functional and methodological control even though the target antigen for aSM is not known.

The similar effects of aThy-1.1 and aGS on cholinergic function need not imply a common target antigen. Nor is it likely that gangliosides are antigenic determinants of Thy-1 antigens. The aGS we used (preparation 2229 supplied by Dr M. M. Rapport) was raised against whole beef brain gangliosides and it is reported to react with GM<sub>1</sub>, G<sub>D1b</sub> and asialo-GM<sub>1</sub>, but not with G<sub>D1a</sub> or G<sub>T1</sub> gangliosides<sup>29</sup>. It is possible that some antibody in this antiserum cross-reacts with a carbohydrate determinant present on rat brain Thy-1 antigen. To test this possibility, we isolated the IgG fraction of the aGS serum on a staphylococcal protein A-Sepharose 4B column<sup>30</sup>. By radioimmunoassay with <sup>125</sup>I-protein A, we find significant binding of aGS2229-IgG to purified rat brain Thy-1 antigen (supplied by Dr A. F. Williams). Consistent with the different carbohydrate composition reported for rat thymocyte Thy-1<sup>23,24</sup>, we are unable to demonstrate binding of aGS2229 immunoglobulin to rat thymocytes by indirect immunofluorescence.

These experiments have tentatively assigned a functional correlate to the Thy-1 antigen. Determination of the mechanisms by which aThy-1.1 causes behavioural alterations remain to be investigated. As full responsiveness was recovered in 3 d or less, it is unlikely that the effects are caused by complement-mediated cytotoxic lesions. More likely is the proposal of Barclay *et al.*<sup>23</sup> and Williams *et al.*<sup>24</sup>, that the protein mediates a common and special cellular function wherever the Thy-1 antigen is found; the carbohydrate, which may vary from one tissue to another, contributes a specificity to the cell's reactions with other cells and with substances in the extracellular environment. The binding of aThy-1.1 to the protein may affect the function of the molecule by steric hindrance of the carbohydrate specifying function, or by causing a conformational constraint in the protein, or by cross-linking the complex into a nonfunctional array in the cell surface.

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## Respiratory oscillations in arterial carbon dioxide tension as a control signal in exercise

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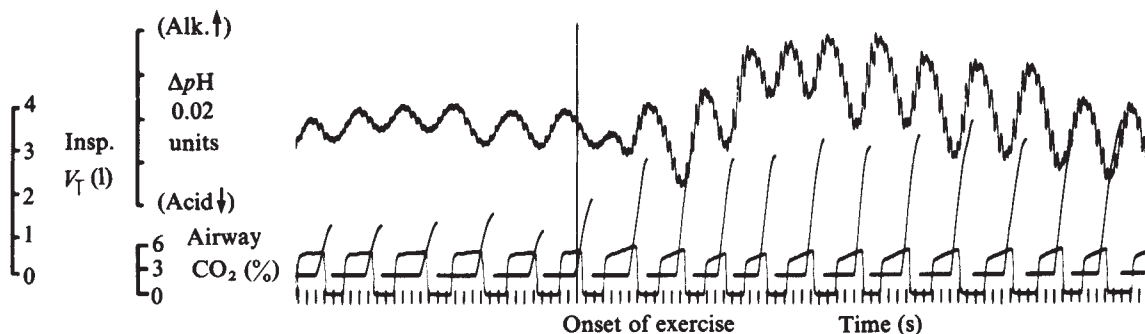
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**We have monitored oscillations in arterial pH (of respiratory frequency) in normal man at rest and during exercise. The pH oscillations are known to reflect respiratory oscillations in arterial carbon dioxide tension generated at the lungs. We have found that the pH oscillations increase in their upslope and downslope during exercise. This means that oscillations in arterial carbon dioxide tension can be considered as a control signal.**

During exercise there is a considerable increase in the ventilation of the lungs which matches the increase in the oxygen requirement of the body and the increase in carbon dioxide production. The ventilatory increase is adequate to keep the levels of oxygen and carbon dioxide reasonably constant in the lungs and also therefore in the arterial blood. How the ventilation is so precisely adjusted to meet the need for oxygen uptake [ $\dot{V}O_2$ ] and carbon dioxide clearance [ $\dot{V}CO_2$ ] has remained obscure in spite of our knowledge of receptors sensitive to altered levels of arterial tensions of both oxygen (the peripheral arterial chemoreceptors) and carbon dioxide (principally the central—medullary—chemoreceptors). Because the arterial levels of oxygen tension [ $P_{aO_2}$ ] and carbon dioxide tension [ $P_{aCO_2}$ ] do not change much in exercise, Yamamoto suggested that  $P_{aCO_2}$  oscillations of respiratory frequency might be present and, theoretically at least, would convey information as to  $\dot{V}CO_2$  (ref. 1). For  $P_{aCO_2}$  oscillations to be a reasonable candidate as a control signal in exercise they must get bigger—or at least steeper. The peripheral arterial chemoreceptors are now known to have a high dynamic sensitivity to  $P_{aCO_2}$  oscillations in the anaesthetised cat<sup>2</sup>, although for technical reasons the  $P_{aCO_2}$  oscillations are measured indirectly, as changes in acidity, with a fast response pH electrode. Although direct measurement with a  $PCO_2$  electrode would be preferable none respond sufficiently fast. Even the fast response pH electrode





**Fig. 1** Photographic record of arterial pH oscillations before and after the onset of exercise (40 W) in a healthy subject (C.B.W.). The records below the arterial pH record, monitored by means of a left brachial artery cannula, are inspiratory (Insp.) tidal volume ( $V_T$ ), the airway carbon dioxide concentration, and lines showing the passage of time in seconds.  $\dot{V}CO_2$  and the average of upslope and downslope of the pH oscillations ( $dpH/dt$ ) increase 2.7 times with exercise.  $\dot{V}CO_2$  increases from 304 to 827 ml min<sup>-1</sup> BTPS;  $dpH/dt$  from  $4.41 \times 10^{-3}$  to  $11.95 \times 10^{-3}$  pH units per second.

systems have so far been highly vulnerable to movement, with excessive electrical noise on any attempt to use them for the indirect measurement of  $P_aCO_2$  oscillations in exercise.

Most of the electrical interference problems have been avoided by linking a fast response pH electrode system to a small radio transmitter, while monitoring arterial pH from subjects exercising on a bicycle ergometer. This has made possible transmission of the signal to a remote receiver. It has therefore been possible to monitor respiratory oscillations of the arterial pH in exercise. We found (Fig. 1) that the pH oscillations become steeper during exercise, as postulated if  $P_aCO_2$  oscil-

lations are to be considered as a possible control signal. The change in the form of the oscillation became apparent within 10 s of the onset of exercise.

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## Spread of activation and desensitisation in rod outer segments

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Visual transduction in vertebrate photoreceptors is thought to involve a diffusible internal transmitter which links photon absorption in disk membranes to the conductance decrease in the plasma membrane of the outer segment<sup>1–5</sup>. The desensitisation of the photoresponse which occurs during and after illumination may also involve a diffusible substance, because a photon absorbed in one disk can desensitise the cell's response to a subsequent photon absorbed in a different disk<sup>6</sup>. Although both activation and desensitisation spread longitudinally from the site of photon absorption, neither spreads over the entire length of the outer segment<sup>7,8</sup>. We report here experiments which indicate that, with dim illumination, both effects decline to be half-maximal in less than 8  $\mu$ m from the site of light absorption.

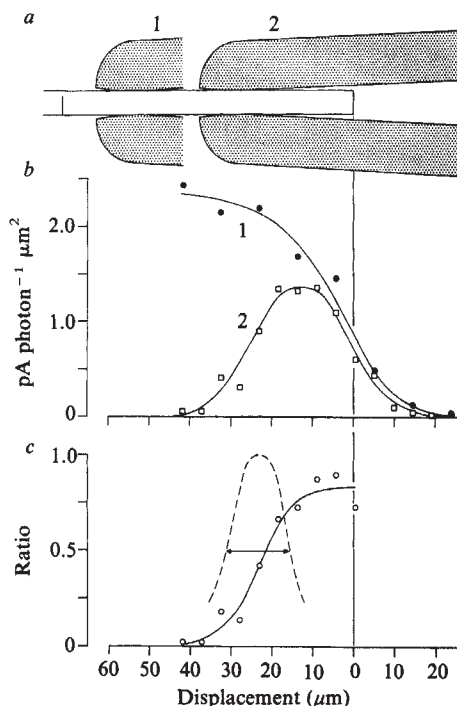
A single toad rod outer segment was drawn into a closely fitting glass pipette by a recently developed technique<sup>9</sup> and its membrane current was measured. Narrow transverse slits of light could then be positioned as desired along the outer segment, allowing investigation of the spread of activation and desensitisation from the site of light absorption.

In Fig. 1b the peak current collected by the pipette, scaled by the flash intensity, is plotted as a function of the displacement of a narrow slit of light from the distal tip, for the cases in which the outer segment was almost completely (●) or only partly (□) sucked into the recording pipette, as indicated schematically in

(a). In this way curve 2 represents a profile of recorded current as the stimulus was moved off the recording pipette, while curve 1 is a control representing the current collected from the same parts of the outer segment when it was almost completely drawn into the pipette. The shapes of the responses were similar at all stimulus positions except when the slit was some distance off the pipette tip. In this case the intensity had to be substantially increased to give a measurable response, which then exhibited a small (less than 0.5 pA) rapid inward component preceding the normal outward current change. This inward transient might be due to the unstimulated part of the outer segment acting as a current sink. When scaled by flash intensity this negative component was very small on the scale of Fig. 1.

This figure illustrates that activation caused by illumination at one point does not spread over the entire outer segment. For example, stimulation of the point on the outer segment 40  $\mu$ m from the distal tip gave a measured response which was about 40 times smaller when that part of the cell was just outside the pipette than when it was inside.

The ratio of the two profiles of recorded current, plotted in Fig. 1c, gives a measure of the current collected by the pipette as the slit is moved off the tip, corrected for non-uniformities in the sensitivity of the outer segment. If the pipette were a perfect step collector of current (that is if it collected all the current from the region of the cell inside the point of narrowest constriction and none from outside), then the profile in Fig. 1c would represent the integral of the membrane current density from the tip of the outer segment to the constriction. Hence the spatial derivative (broken curve) would represent the spread of activation from the narrow line source of illumination; its width at half-height is 15  $\mu$ m (arrow). This profile is considerably broader than the spread of the stimulating light. Unfortunately we have no direct measure of the collecting efficiency of the pipette, as would be needed to correct the spread function, and we can say only that the curve probably represents an upper limit to the actual spread



**Fig. 1** Spread of activation in a rod outer segment from *Bufo marinus*. *a*, Scale diagram of the outer segment almost completely (1) and partly (2) drawn into pipette. *b*, Recorded current, divided by flash intensity, as a function of displacement of a transverse slit from the tip of the outer segment, for the two positions of the cell. Nominal slit width 6.9  $\mu\text{m}$ . Saturating response of the rod to diffuse light was 18 pA. In this and subsequent figures 20 responses were averaged and flash intensity was adjusted to give approximately 2 pA response; 20-ms flash; 500-nm light plane polarised perpendicular to the long axis of the outer segment; temperature 20°–24 °C. *c*, Ratio of profiles in (*b*);  $\circ$  are the ratio of squares ( $\square$ ) to the curve 1 near the filled circles ( $\bullet$ ); broken curve is the normalised spatial derivative of solid curve. The right hand parts of the curves in (*b*) do not coincide, possibly because of slight movement of the tip of the cell during the experiment. For this reason the ratio has not been plotted beyond 0  $\mu\text{m}$ .

of activation. We believe that this profile has not been substantially narrowed by the presence of current sinks in the unilluminated region because, as discussed above, such sink currents appear to be small. Our upper limit is in broad agreement with a previous estimate of spread in rat rods of 12  $\mu\text{m}$  from the point of absorption<sup>7</sup>.

A lower limit can be obtained from the ratio of the amplitude of the single photon event of about 1 pA (ref. 10) to the amplitude of a saturating response of about 30 pA (ref. 9). This indicates that at least 1/30 of the outer segment plasma membrane must be affected by a single isomerisation, so that for an outer segment 60  $\mu\text{m}$  long activation must spread over a longitudinal region of at least 2  $\mu\text{m}$ , corresponding to 1  $\mu\text{m}$  either side of the point of photon absorption.

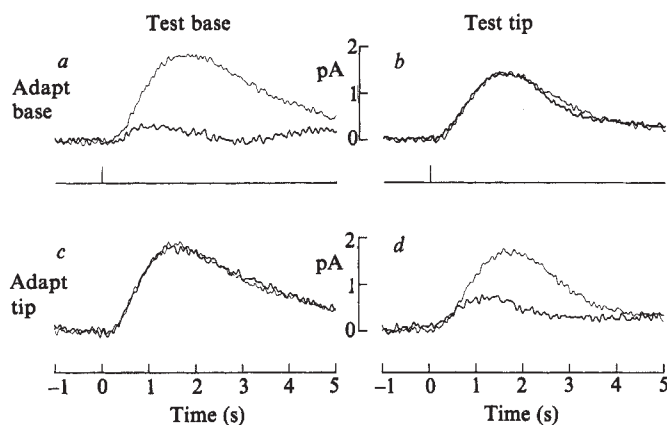
Figure 2 shows that desensitisation is also localised. Narrow slits were used for both the brief test flash and the steady adapting light, and each was presented near either the base or tip of the outer segment. The heavy traces are averaged responses to the test flashes presented during adapting illumination, while the lighter traces are controls in which the same flashes were presented in darkness. In (*a*) and (*d*) the test and adapting slits were superimposed at the base and tip respectively, while in (*b*) and (*c*) the test and adapting slits were at opposite ends of the outer segment, separated by approximately 30  $\mu\text{m}$ . When they were superimposed the response to the test flash was substantially smaller and faster, but when they were at opposite ends of

the outer segment the test response was unaffected by the adapting light. This experiment shows that a light which locally desensitises by three- or fourfold has negligible adapting effect at a distance of 30  $\mu\text{m}$ . On all occasions when substantial desensitisation was observed it was accompanied by accelerated response kinetics, suggesting that the reduction in response amplitude was not due solely to local saturation of light-sensitive channels.

The extent of spread of desensitisation is examined in another cell in Fig. 3. Here desensitisation is expressed as the ratio of sensitivity in darkness to sensitivity in the presence of the steady adapting slit, rather analogous to a plot of threshold elevation in psychophysical experiments. Assuming that the measured profile is symmetrical (broken curve in Fig. 3), it has a width at half-height of 8  $\mu\text{m}$  (arrow). The desensitisation has been plotted in this way in an attempt to obtain a linearised measure of the effect of adapting light. With diffuse illumination, toad rod sensitivity shows Weber–Fechner behaviour<sup>6,11,12</sup>, defined by

$$\frac{S_F^D}{S_F} = 1 + \frac{I}{I_0} \quad (1)$$

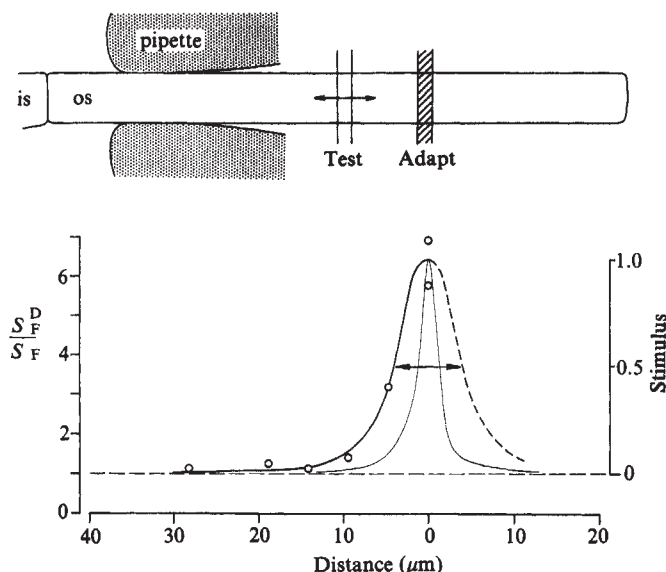
where  $S_F$  is the sensitivity in the presence of background intensity  $I$ ,  $S_F^D$  is the sensitivity in darkness and  $I_0$  is the intensity that halves sensitivity. Note that the term  $(S_F^D/S_F - 1)$  is directly proportional to adapting intensity. If we assume that light generates a diffusible substance whose concentration rises linearly with intensity, and that desensitisation at any point on the outer segment depends on the local concentration of this substance, then we can interpret the profile above the line  $S_F^D/S_F = 1$  in Fig. 3 to represent the spread of desensitising substance. For comparison the measured light spread is indicated by the narrower curve in the figure; it has a width at half-height of only 2.6  $\mu\text{m}$ .



**Fig. 2** Localisation of desensitisation. The test and adapting stimuli were narrow slits which could be independently positioned near either the tip or the base (as indicated) of an outer segment drawn fully into the pipette; the two slit positions were separated by about 30  $\mu\text{m}$ . In each part of the figure the heavier trace is the averaged response to the test flash in the presence of the steady adapting slit, and the lighter trace is the control in darkness. Identical stimuli were used throughout; adapting slit gave steady response of about 3 pA at both positions; nominal slit widths 6.9  $\mu\text{m}$ . Saturating response to diffuse light was 20 pA. Responses low-pass filtered at 10 Hz.

Some justification for the linearising procedure adopted in the previous paragraph comes from a comparison between the effects of local and uniform illumination. In Fig. 3 the adapting slit delivered about 7 photoisomerisations per s and the area under the profile of  $(S_F^D/S_F - 1)$  was 54  $\mu\text{m}$ . If the same number of photoisomerisations were spread uniformly over the whole outer segment length of 60  $\mu\text{m}$  then the average value of  $(S_F^D/S_F - 1)$  would be 54  $\mu\text{m}$ /60  $\mu\text{m}$  = 0.9. Thus the intensity





**Fig. 3** Spread of desensitisation. Top: diagram of outer segment and light slits on the same scale as the lower abscissa. Bottom: desensitisation, expressed as dark sensitivity ( $S_F^D$ ) divided by sensitivity ( $S_F$ ) in the presence of the adapting light (left-hand ordinate, open circles; heavy curve fitted to the points by eye, and assumed to be symmetrical to obtain the broken extension), as a function of distance between the steady adapting slit and the test flash slit (abscissa). The position of the steady adapting light was fixed (0  $\mu\text{m}$  on abscissa) and the test flash was moved to different positions on the outer segment. The thin line shows the normalised intensity (right-hand ordinate) of the adapting slit, determined in another experiment by moving the image of the slit across a small aperture placed in the microscope eyepiece. Both slits had a nominal width of 1.7  $\mu\text{m}$ . The steady light had a nominal intensity of 4.6 photons  $\mu\text{m}^{-2} \text{s}^{-1}$ , and produced a mean response of 2.2 pA. Saturating response to diffuse light was 16.4 pA.

$I_0$  of diffuse light required to halve the sensitivity (that is,  $S_F^D/S_F - 1 = 1$ ) would be  $7/0.9 = 7.8$  photoisomerisations per s. This is in reasonable agreement with measured values of about 4 photoisomerisations per s<sup>6,11,12</sup>.

Preliminary experiments indicate that the integration time<sup>13</sup> of desensitisation resulting from flash illumination is about 3.5 s. Applying this value and equation (1) to the cell in Fig. 3 we estimate that, during this integration time, a single photoisomerisation should on average reduce sensitivity by about 18% (that is  $S_F/S_F^D \approx 0.82$ ) at the site of light absorption.

We conclude that the spread of activation and desensitisation is restricted mainly to a distance of 5–10  $\mu\text{m}$  along the outer segment from the site of photon absorption. The extent of spread of desensitisation lies between our upper and lower estimates for spread of activation, and it is possible that both effects spread to the same extent, as would be the case if the same diffusible substance were ultimately responsible for both processes.

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## Localisation of substance P immunoreactivity in amacrine cells of the retina

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Since its actions on smooth muscle were first described by von Euler and Gaddum<sup>1</sup>, substance P has been isolated and characterised as an undecapeptide<sup>2,3</sup>. The distribution of substance P-like immunoreactivity in both the central and peripheral nervous systems<sup>4,5</sup> and its reported excitatory action on neurones implicate substance P as a possible neurotransmitter and/or neuromodulator<sup>6,7</sup>. Previous studies using bioassay<sup>8,9</sup> and radioimmunoassay<sup>10,11</sup> techniques have reported substance P in retinal extracts. Here we describe the specific immunohistochemical localisation of substance P-like immunoreactivity in a population of morphologically distinct amacrine cells of the pigeon retina. The localisation of substance P-like immunoreactivity and the recent localisation of enkephalin-like<sup>12,13</sup> and somatostatin-like immunoreactivity<sup>14</sup> in other types of amacrine cells suggest that the neuropeptides have a specific role in retinal function.

Details of the methods used for localisation are described in the legend to Fig. 1. Substance P-like immunoreactivity was observed only in the inner nuclear and inner plexiform layers of the retina with both the indirect immunofluorescence and immuno-peroxidase bridge procedures. Specific substance P-like immunoreactivity was observed in the somata of amacrine cells usually located at the border of the inner nuclear and inner plexiform layers (Figs 1a, 2). No immunoreactive somata were observed in the outer nuclear layer, the distal inner nuclear layer or the ganglion cell layer. Muller cells were not immunoreactive. Immunoreactive cell processes were observed only in the inner plexiform layer.

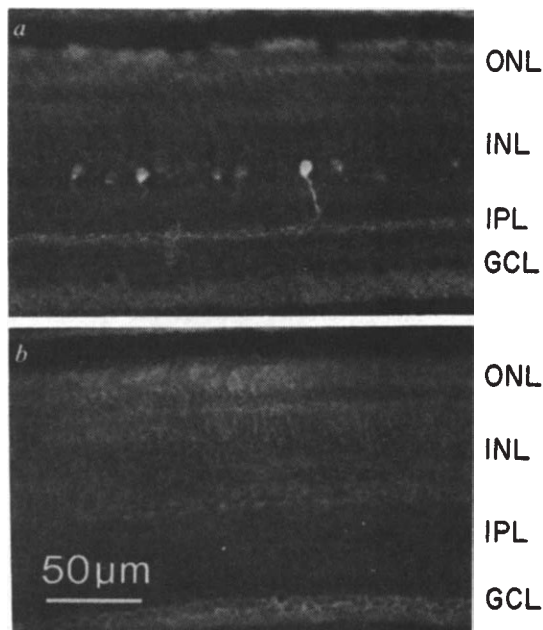
The immunoreactive amacrine cells have a single process, which descends directly into the inner plexiform layer and ramifies in a narrow band of the inner plexiform layer corresponding to lamina 3 of Cajal<sup>15</sup>. Substance P-like immunoreactivity was observed in both central and peripheral retinal regions. The immunoreactive amacrine cells in the tangential sections were about 7  $\mu\text{m}$  in diameter and were distributed throughout the retina. Relative to other retinal regions, a greater number of immunoreactive somata were located in the inferior retina and a smaller number of immunoreactive somata were located within the red field of the superior retina.

The staining for substance P seems to be specific, as it was eliminated by incubation of the retinal sections in substance P monoclonal antiserum preabsorbed with 10  $\mu\text{M}$  synthetic substance P (Fig. 1b). Moreover, retinal sections incubated in substance P antiserum preabsorbed with 10  $\mu\text{M}$  synthetic neurotensin, Leu<sup>5</sup>-enkephalin, Met<sup>5</sup>-enkephalin, somatostatin or thyrotropin-releasing factor demonstrated positive immunoreactivity in the same type of amacrine cell, indicating that the substance P antiserum did not react with these other peptides in our incubation conditions. Incubation of retinal sections in either normal or preabsorbed antisera demonstrated nonspecific immunoreactivity in a few ganglion cells located in the peripheral retina.

The present study demonstrates a specific population of substance P-like immunoreactive amacrine cells and their processes in the avian retina. The consistent size of their somata,

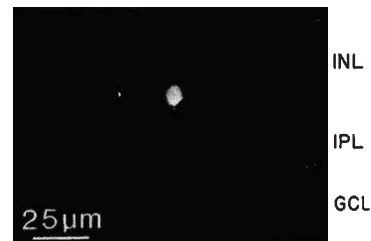
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their location in the proximal inner nuclear layer and arborisation pattern in lamina 3 of the inner plexiform layer suggest that neither bipolar cells nor displaced ganglion cells contain substance P-like immunoreactivity<sup>15,16</sup>. The profile of the substance P-like immunoreactive cell is similar to amacrine cell type 'J' described by Cajal<sup>13</sup> from Golgi material. On the basis of somatic location and arborisation patterns, the substance P-like immunoreactive cells do not seem to overlap with the indoleamine<sup>17</sup> or dopamine<sup>18</sup> containing amacrine cells.



**Fig. 1** *a*, Low power photomicrograph of a retinal section incubated in substance P antiserum demonstrating substance P-like immunoreactivity in an amacrine cell and in processes located in the inner plexiform layer. Several other substance P-like immunoreactive somata occur in a different plane of focus. *b*, Low power photomicrograph of a retinal section incubated in substance P antiserum which had been preabsorbed with 10  $\mu$ M synthetic substance P, demonstrating lack of substance P-like immunoreactivity. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Retinal sections for Figs 1 and 2 were prepared as follows. Adult white Carneaux pigeons were deeply anaesthetised with intramuscular ketamine and perfused through the left ventricle with 6% dextran in 0.1 M phosphate buffer (pH 7.2) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). During the perfusion the eyes were injected with the fixative. They were postfixed for 4–6 h at 4 °C and stored overnight in 30% sucrose phosphate buffer at 4 °C. Sections were cut perpendicular to the vertical axis of the eye (horizontal plane) at 12  $\mu$ m with a cryostat or tangential to the retinal surface at 30  $\mu$ m with a freezing microtome. They were processed for immunohistochemical staining using either the indirect immunofluorescence or the immunoperoxidase bridge techniques previously described<sup>13</sup>. Sections were incubated in substance P antiserum or substance P antiserum which had previously been absorbed with either 10  $\mu$ M synthetic substance P, neurotensin, Leu<sup>5</sup>-enkephalin, Met<sup>5</sup>-enkephalin, somatostatin or thyrotropin-releasing factor at a dilution of 1:1,000 for 12–48 h at 4 °C. These experiments were replicated with at least 12 retinas except for the preabsorption series of neurotensin, Leu<sup>5</sup>-enkephalin, Met<sup>5</sup>-enkephalin, somatostatin and thyrotropin-releasing factor, which were replicated with two pigeon retinas.

The localisation and distribution of substance P-like immunoreactive amacrine cells differ from those of enkephalin-like immunoreactive amacrine cells in the avian retina<sup>13</sup>. Enkephalin-like immunoreactivity has been observed in many small amacrine cells, giving rise to a diffuse plexus of processes which ramify in laminae 1 and 3–5 of the inner plexiform layer. In contrast, the substance P-like immunoreactive amacrine cells, although also small, ramify in lamina 3 of the inner plexiform layer. The enkephalin-like immunoreactive amacrine cells are distributed throughout the entire retina, are most numerous in the red field of the superior retina and may be related to the total number of cells in the inner nuclear layer<sup>19</sup>. The distribution of substance P-like immunoreactive amacrine cells, however, does



**Fig. 2** High power photomicrograph demonstrating a substance P-like immunoreactive amacrine cell.

not seem simply to be related to the total number of cells in the inner nuclear layer in as much as the greatest numbers of substance P-like immunoreactive cells are in inferior retinal regions and not the red field. The differential distribution of substance P-like immunoreactivity suggests a histochemical as well as a morphological basis for classification of retinal amacrine cells. Amacrine cells have been classified on the basis of their histochemical characteristics and their differential uptake systems for  $\gamma$ -aminoglutaric acid, glycine, choline or dopamine<sup>20–23</sup>. However, the total number of amacrine cells and the distribution of subclasses of amacrine cells in the retina are unknown. Therefore, it is not yet possible to compare the number of substance P-like and enkephalin-like immunoreactive amacrine cells with other histochemically distinct subclasses of amacrine cells.

Recently, several neuropeptides have been found in the retina of different vertebrate species using radioimmunoassay techniques<sup>24–28</sup>. Substance P-like, enkephalin-like, neurotensin-like and somatostatin-like immunoreactivity have been localised in distinct classes of amacrine cells of different vertebrate retinas using the immunohistochemical technique (refs 12–14 and unpublished observations).

In conclusion, specific substance P-like immunoreactivity occurs in a distinct class of retinal amacrine cells. The specific functional role of substance P or any other neuropeptide in the retina is unknown although their localisation in selected populations of retinal cell types suggest they have unique roles in retinal processing.

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## Removal of the synaptic target permits terminal sprouting of a mature intact axon

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When the central nervous system (CNS) develops, neurones send out axons to make contact with appropriate synaptic target cells and then stop growing. If its usual target is missing, an axon may continue to grow until it synapses with a suitable but inappropriate target<sup>1</sup>. This suggests that contact with a synaptic target is important in stopping axonal growth during development. Many classes of neurone in the adult CNS retain a capacity to grow towards denervated targets<sup>2-5</sup>, but it is not known whether the synaptic contacts established during development continue to regulate the growth of individual mature, intact axons. This has been a difficult problem to investigate; in vertebrates most studies necessarily involve large populations of neurones, and the most direct approach, removal of a synaptic target, usually damages many neurones, including the axons that are to be studied. We report here a demonstration of target cell influences on the growth of a single mature, intact axon in the CNS of the leech by selectively destroying the axon's synaptic target without injuring the axon itself. Target removal, which itself does not trigger sprouting of intact axons, permits the intact axon to grow at its tip in response to injury of other axonal branches of the same cell.

The system of S-interneurones in the leech has been particularly useful for studies of axonal growth and synapse regeneration<sup>6-8</sup>. In each of the 21 segmental ganglia there is one S-cell; it extends an axon both anteriorly and posteriorly about halfway along the connectives (the axon bundles that link adjacent ganglia) to make an electrical synapse exclusively with the tip of the next S-cell axon<sup>8</sup>. For brevity, we shall use the 'target' of the S-cell axon to denote the neurone with which that axon is synaptically linked. In this sense, each of the two synaptically coupled S-cell axons is the target of the other.

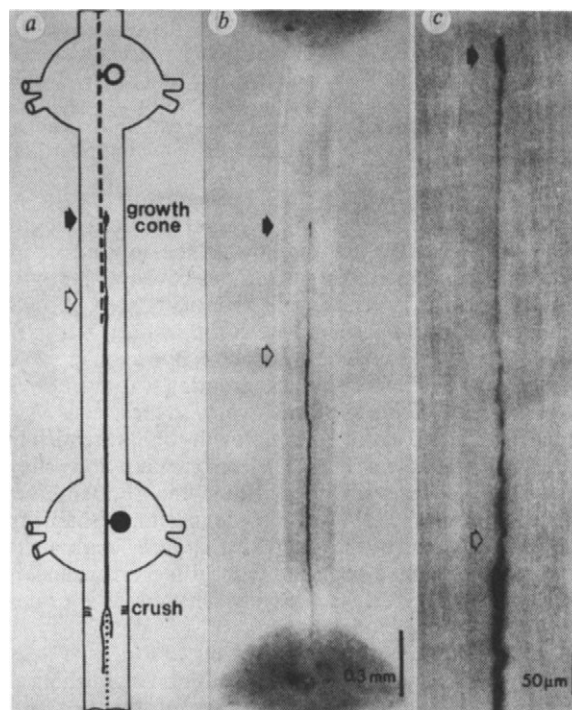
To test whether contact with a synaptic target has any influence on mature intact axons, we selectively killed single S-cells in the nervous system of an otherwise intact leech by injecting the soma of the target cell with a protease (Type VIII, Sigma)<sup>9,10</sup>. This approach was taken because it is not possible to eliminate the target by removing its soma, as the S-cell axon can survive for weeks severed from its cell body. Protease-injected S-cells were rapidly and completely destroyed, with no direct electrophysiologically or morphologically measurable effects on other cells<sup>9,10</sup>. The long-term effects on the uninjured S-cells adjacent to the killed cell were followed by dissecting chains of ganglia from 4 days to 6 months after the operation, impaling the S-cells with microelectrodes, and injecting them with horseradish peroxidase (HRP), a marker that does not cross the S-cell synapse but fills the injected cell<sup>8</sup>, or with Lucifer Yellow, a fluorescent dye that does cross between electrically coupled cells<sup>10,11</sup>, or with both. We studied 15 neurones in 14 preparations using electrophysiological methods, and stained and examined them with light and electron microscopy.

The S-cell axons that had been coupled to the killed cells were apparently unaffected by the loss of this synaptic contact. The neurones maintained full-calibre axons that extended to the original site of synapse with the killed cell, about halfway along the connectives, and they showed no signs of growth or retraction. The intact axons did not develop recognisable growth cones filled with characteristic smooth membranous reticulum<sup>8,12</sup>. Furthermore, the free axons apparently did not form synapses with other axons in the connectives. Lucifer Yellow dye provides one test for electrical synapses that resemble those

made between normal S-cells. When injected into either a normal or a regenerated S-cell, the dye readily diffuses down the axons and selectively fills the adjacent S-cells, crossing into them at the electrical synapse (S.A.S. and K.J.M., in preparation). In contrast, Lucifer Yellow injected into S-cells that were adjacent to a killed cell stopped abruptly at the tip of the free, intact axon midway along the connectives, and filled no other axons (S.A.S. and K.J.M., in preparation). Moreover, no structures resembling electrical<sup>8</sup> or chemical<sup>13</sup> synapses were seen near the tips of such intact axons when viewed in the electron microscope. Thus, an S-cell axon does not seem to be affected when its unique synaptic target is selectively destroyed.

One obvious difference between neurones in developing nervous systems and those in adults that could account for the failure of the adult neurone to respond to the loss of a target is that in the adult the neurone is no longer growing when its target is destroyed. To prompt the adult S-neurone to mobilise its cellular machinery for growth, we injured the cell by severing one of its two principal axons. The target of the neurone's other, intact axon was then removed by injecting that target cell with protease (Fig. 1a). A total of 22 intact axons of injured neurones in 12 preparations were examined from 9 days to 6 months after surgery. Identical results were obtained whether a cell's anterior target or its posterior target S-cell was killed.

The severed axon of these cells regenerated normally<sup>8</sup> to form an electrical synapse selectively with the tip of the adjacent S-cell axon in about 1 month, but the response of the intact axon was surprising. Ordinarily, when one S-cell axon is severed, the cell's other, intact axon is not measurably affected by the lesion;



**Fig. 1** An intact axon can be made to sprout when its synaptic target is selectively removed. The neurone in the ganglion at top (anterior) was injected with protease, which destroyed the entire cell (dashed line and open circle in *a*). A distant axon of the adjacent S-cell was then severed by crushing the connective (crush). The injured axon regenerated in a normal fashion across the crush and along its severed distal stump (dotted line, *a*); the intact axon of that cell, which had been in synaptic contact with the killed cell near the connective midpoint (open arrow), sprouted a fine process tipped with a growth cone (solid arrow). In *b* the sprouting neurone was marked by intracellular injection of horseradish peroxidase 25 days after its posterior axon (not visible) had been severed. *c*, The sprouted axon is seen at higher magnification, showing the growth cone (solid arrow) extending from the original axon tip (open arrow). This tissue was not cleared so that it could be subsequently examined in the electron microscope.

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**Fig. 2** The axon that sprouts in response to the loss of its target grows along the path of the destroyed cell. The sprouted neurone was marked with intracellular injection of horseradish peroxidase 38 days after the adjacent S-cell had been killed. The electron micrograph in *a* shows a cross-section of the connective cut through the growth cone of the sprouted axon, which is located at the position in the middle connective (Faivre's nerve) that is normally occupied by the killed cell. The growth cone contains much smooth membranous reticulum (arrowheads in *b*, an enlargement of boxed region of *a*) characteristic of the tips of growing axons.

it remains in synaptic contact with its target and does not grow<sup>8</sup>. In striking contrast, the intact axon of the injured neurone sprouted at its tip when its target was destroyed with protease. Most intact axons of injured S-cells eventually grew about half the distance from the original axon tip, which was clearly recognisable (Fig. 1), to the next ganglion; one axon grew as far as the adjacent ganglion. Terminal sprouting was most obvious in cells examined 3–6 weeks after surgery. In these cells a single fine process, enlarged at the tip to a growth cone, extended from the end of the intact axon (Fig. 1). Electron microscopy showed that the growth cones were filled with a smooth membranous reticulum and that they followed a route indistinguishable from that formerly occupied by the killed S-cell (Fig. 2). Although this route might simply have been the path of least resistance, the possibility remains that it was actively selected, as is evidently done by regenerating axons<sup>8</sup>.

The sprouted axons stopped growing during the second month after surgery, apparently without establishing any synaptic contacts. Electron micrographs of the tips of the sprouted axons showed that the growth cone together with its content of smooth membranous reticulum disappeared, and that no structures resembling chemical or electrical synapses had formed. Furthermore, Lucifer Yellow injected into sprouted S-cells filled the intact axon to its tip without passing into other axons; this paralleled HRP labelling of the same cells (S.A.S. and K.J.M., in preparation). Under the present circumstances, the sprouted S-cells may have failed to establish synapses in the connectives simply because unoccupied targets were not available. Had the connective itself been injured, the sprouted axons may then have formed synapses with alternative targets<sup>5,14</sup>.

In most preparations examined more than 3 months after surgery, the tip of the S-cell axon was not as fine and long as in preparations examined at earlier times, suggesting that sprouts may begin to retract if they fail to find a suitable target. However, we have seen no evidence of a complete retraction of

the sprouts; even at the longest times it was clear that a sprout extended from the original tip of the axon, which remained distinct.

Thus, removal of the synaptic target of a mature, intact axon in the CNS of the leech permits the axon to sprout if the neurone itself has been triggered to grow by direct injury to another of its branches. Neither simple elimination of the synaptic target nor injury to another axon of the cell is sufficient to induce sprouting, although if new targets could be made available the neurone might sprout<sup>1,3</sup>. We do not know what aspect of cellular injury prompts terminal sprouting of an intact, denervated axon, nor do we know the signal that stops axonal growth, as the axon does not form synapses. It might be that growth stops when the cell reaches some maximal arborisation, or that all growth ceases soon after the severed axon stops regenerating. The findings reported here suggest the possibility that axonal injury triggers general growth processes in the damaged neurone, like those in developing neurones, expressed only by axonal branches that have lost contact with their targets. Although it is not known how the target exerts its effects on the axon, whether by impulse activity across the synapse, transfer of molecules between the coupled cells, direct physical contact, or some other means, it is clear that in the adult nervous system synaptic contacts can inhibit the growth of intact axons.

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## Pirenzepine distinguishes between different subclasses of muscarinic receptors

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Some antagonists exhibit tissue selectivity in their pharmacological antagonism of muscarinic responses<sup>1</sup>. However, the affinity constants for equilibrium binding of classical antagonists to muscarinic receptors in subcellular preparations have shown only small variations in different peripheral tissues and regions of the brain<sup>2–6</sup>. The binding curves do not deviate significantly from the simple Langmuir isotherm, indicating apparent homogeneity of the receptor population in any given region<sup>5–8</sup>. In contrast, heterogeneity has been detected by agonist binding studies<sup>8,9</sup> but this may arise from different environmental or coupling restraints on the agonist-induced conformational change<sup>10,11</sup> and cannot be taken as evidence for different receptor subtypes. We report here binding studies using a new anti-muscarinic drug, pirenzepine, in which we found heterogeneity of binding that correlates well with the pharmacological activity.

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Pirenzepine (LS 519 C12), (Fig. 1) inhibits gastric secretion in animals<sup>12</sup> and man<sup>13-15</sup>, and there is strong evidence that this effect is governed by an antimuscarinic mechanism<sup>16,17</sup>. It also antagonises the cholinergically stimulated contraction of the smooth muscle of the ileum<sup>18</sup> and urinary bladder<sup>19</sup>, blocks salivation, and causes tachycardia<sup>19</sup>. However, in contrast to the action of classical antimuscarinic drugs, there is a wide disparity between the doses of pirenzepine needed to evoke these different effects; whilst effects on gastric secretion are seen at very low doses, higher doses are needed to inhibit salivation, and only with very high doses are effects seen on the smooth muscle of the stomach wall and on the heart<sup>19,20</sup>. Central effects are not significant since the drug is hydrophilic<sup>21</sup> and therefore does not cross the blood barrier to any appreciable extent<sup>22</sup>. In the present study the binding of pirenzepine to muscarinic receptors in homogenates of several peripheral tissues and in different regions of the rat brain has been compared to that of a classical muscarinic antagonist, *N*-methylscopolamine (NMS).

The binding of NMS was determined directly using the radiolabelled ligand <sup>3</sup>H-NMS (refs 7, 9) and representative binding curves in the sublingual gland and atria are shown in Fig. 2. There is only a small difference in the two estimates of the <sup>3</sup>H-NMS dissociation constant in the two tissues and in all the areas examined the <sup>3</sup>H-NMS binding curves are adequately

**Table 1** Pirenzepine and NMS binding in peripheral organs and in brain

| Region                  | Pirenzepine<br>IC <sub>50</sub> cor(M) | <i>H</i> | NMS<br>K <sub>d</sub> (M) | <i>H</i> |
|-------------------------|----------------------------------------|----------|---------------------------|----------|
| Brain                   |                                        |          |                           |          |
| Hippocampus             | 3.4 × 10 <sup>-8</sup>                 | 0.80*    | 2.7 × 10 <sup>-10</sup>   | 1.05     |
| Cerebral cortex         | 5.1 × 10 <sup>-8</sup>                 | 0.73*    | 3.6 × 10 <sup>-10</sup>   | 1.00     |
| Medulla-pons            | 4.1 × 10 <sup>-7</sup>                 | 0.97     | 4.5 × 10 <sup>-10</sup>   | 1.04     |
| Glands                  |                                        |          |                           |          |
| Sublingual              | 1.1 × 10 <sup>-7</sup>                 | 0.72*    | 3.0 × 10 <sup>-10</sup>   | 0.91     |
| Submandibular           | 1.3 × 10 <sup>-7</sup>                 | 0.78*    | 2.5 × 10 <sup>-10</sup>   | 1.01     |
| Lacrimal                | 2.0 × 10 <sup>-7</sup>                 | 0.97     | 3.2 × 10 <sup>-10</sup>   | 1.04     |
| Parotid                 | 2.9 × 10 <sup>-7</sup>                 | 0.97     | 2.6 × 10 <sup>-10</sup>   | 0.90     |
| Heart and smooth muscle |                                        |          |                           |          |
| Atria                   | 8.3 × 10 <sup>-7</sup>                 | 0.96     | 5.9 × 10 <sup>-10</sup>   | 0.96     |
| Ileum wall              | 7.9 × 10 <sup>-7</sup>                 | 0.96     | 6.7 × 10 <sup>-10</sup>   | 0.96     |
| Stomach wall            | 1.1 × 10 <sup>-6</sup>                 | 0.98     | 5.3 × 10 <sup>-10</sup>   | 1.08     |

All manipulations and assays were performed in 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 20 mM HEPES (pH 7.5). The salivary glands, lacrimal gland and atria were homogenised initially in buffer (Sorvall Omnimixer, setting 4, 60 s, 0 °C). The ileum and stomach wall were dissected free of mucosa and homogenised (Ultra-Turrax, setting 6, 2 × 30 s, 0 °C). All initial homogenates of peripheral organs were further homogenised (Potter-Elvehjem, 20 strokes, 520 r.p.m., 0 °C) and filtered through cheesecloth. It was found that the presence of the proteolytic inhibitor, phenylmethylsulphonylfluoride (1 mM) had little effect on the receptor binding properties. The brain regions were only subjected to the Potter homogenisation. For the binding assays the homogenates in appropriate dilutions (~1 mg protein ml<sup>-1</sup>) were preincubated for 10 min at 30 °C before addition to microcentrifuge tubes containing labelled ligands with or without unlabelled ligands. Incubation was carried out for 20 min (at which time, binding was at equilibrium) at 30 °C and was terminated by centrifugation as previously described<sup>7</sup>. Assays were carried out in quadruplicate and the non-specific binding defined as the radioactivity bound or entrapped in the pellet when the incubation medium contained 10<sup>-6</sup> M 3-quinuclidinyl-benzilate. Binding curves for NMS were measured directly with the radio-labelled ligand. Binding curves for pirenzepine were derived indirectly from competition studies against 3 × 10<sup>-10</sup> M <sup>3</sup>H-NMS (peripheral organs), or 10<sup>-9</sup> M <sup>3</sup>H-propylbenzylcholine (brain regions) as described previously<sup>7</sup>. K<sub>d</sub> for NMS were estimated from <sup>3</sup>H-NMS saturation curves according to a one-site model using non-linear least squares regression analysis<sup>7</sup>. The IC<sub>50</sub> values for pirenzepine binding were corrected for the radioligand occupancy shift according to IC<sub>50</sub>cor = IC<sub>50</sub>/(1 + [C]/K<sub>d</sub>), where K<sub>d</sub> and [C] represent the dissociation constant and the concentration of the radioligand respectively. *H* values were calculated by linear regression analysis and analysed for statistically significant deviation from unity. The data are, in general, the mean of at least two independent measurements which differed in IC<sub>50</sub> value by less than 20%.

\* Significantly different from 1 (*P* < 0.05).

described by a simple Langmuir isotherm having Hill coefficients (*H*) close to, or not significantly different from 1.0 (Table 1).

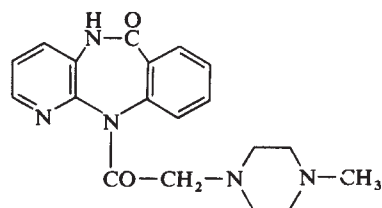
The binding of pirenzepine was determined by the inhibition of receptor-specific binding of <sup>3</sup>H-propylbenzylcholine or <sup>3</sup>H-NMS (refs 7, 9). Occupancy concentration curves for pirenzepine binding in preparations from the cerebral cortex, sublingual gland, parotid gland and atria are shown in Fig. 2. Experimental details are given in the legend to Table 1. It is clear that there are large differences in the concentrations of pirenzepine giving 50% occupancy of the receptors in the various regions. The pirenzepine binding curves in the parotid and atria have *H*-values close to 1.0, whereas the binding curves for pirenzepine in the cortex and sublingual gland deviate significantly from unity (Fig. 2, Table 1) suggesting the presence of sites of more than one affinity<sup>8,9</sup>. Nonlinear least-squares regression analysis shows that the presence of two populations of sites having dissociation constants (K<sub>d</sub>) of 2.5 × 10<sup>-8</sup> M and 0.7–1.0 × 10<sup>-6</sup> M are needed to adequately account for these flat curves. Preliminary direct binding experiments also show that low concentrations of <sup>3</sup>H-pirenzepine bind specifically to a high affinity population of sites in the cortex, with K<sub>d</sub> of 2.5 × 10<sup>-8</sup> M, thereby confirming the indirect measurements (R.H. *et al.*, unpublished results). The pharmacological specificity and the binding properties of the high affinity <sup>3</sup>H-pirenzepine sites suggest that they are a sub-class of those labelled by <sup>3</sup>H-NMS.

Table 1 summarises a series of pirenzepine binding measurements. The lowest IC<sub>50</sub> values for pirenzepine were found in the hippocampus and cerebral cortex; both areas exhibited heterogeneous binding curves. Intermediate values were found in the medulla-pons, the salivary glands and the lacrimal gland. The parotid and lacrimal glands appear to contain relatively uniform populations of pirenzepine sites, with K<sub>d</sub> of 2–3 × 10<sup>-7</sup> M, whilst the sublingual and submandibular glands gave *H* values significantly less than 1.0, and appear to contain a small proportion of high affinity sites. Heart and smooth muscle were found to contain a homogeneous, low affinity population of pirenzepine binding sites (K<sub>d</sub> ≈ 1 × 10<sup>-6</sup> M). All the pirenzepine binding data are compatible with the presence of three sites with K<sub>d</sub> ~ 2 × 10<sup>-8</sup> M, 2 × 10<sup>-7</sup> M and 1 × 10<sup>-6</sup> M respectively; an overall variation of 50-fold. In contrast, the affinity for <sup>3</sup>H-NMS varied by less than a factor of 3 and further confirms the lack of selectivity for binding of classical antagonists to muscarinic receptors from different tissues; again the lowest values were found in heart and smooth muscle with somewhat higher values in the glands and brain, perhaps demonstrating some analogy with the distribution of pirenzepine affinity constants.

The *in vitro* binding profile of pirenzepine in the peripheral organs strikingly parallels the selectivity of the pharmacological profile of the drug, both in animal tests<sup>19,20</sup> and in clinical trials<sup>14,15,23-25</sup>; even very high doses do not cause tachycardia and only occasionally are mild and transient 'dry mouth' effects reported. We have not yet been able to determine the pirenzepine binding properties of the muscarinic receptors in the parietal cells of the gastric mucosa because of proteolysis and the very low concentration of binding sites.

Whilst muscarinic agonists also exhibit heterogeneity of binding, it does not appear that pirenzepine has agonist or partial agonist activity in pharmacological studies, and in addition, in the brain, the affinity profile of pirenzepine is actually opposite to that of the agonists, which show predominantly high

**Fig. 1** Formula of pirenzepine.



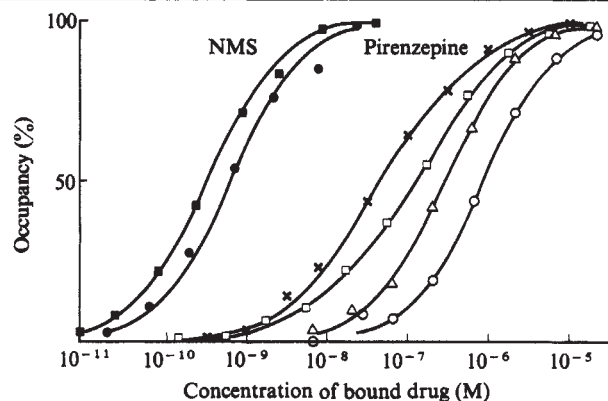


Fig. 2 Occupancy concentration curves for the binding to muscarinic receptors in different tissues of NMS (■ sublingual gland, ● atria) and pirenzepine (× cerebral cortex, □ sublingual gland, △ parotid gland, ○ atria) at 30°C. Experimental details as in the legend to Table 1.

affinity binding in the medulla-pons, and low affinity binding in the hippocampus<sup>5</sup>. Moreover, preliminary experiments show that the binding of pirenzepine is not affected by guanosine triphosphate in tissues, such as heart, where muscarinic agonist binding is modulated by guanine nucleotides<sup>26</sup>. Such behaviour is characteristic of an antagonist.

Previous evidence has favoured the hypothesis that the receptor subtypes detected by agonists do not differ in their intrinsic structure but rather in the molecules associated with them; this could equally be the explanation for the subtypes detected by pirenzepine, but we cannot yet exclude the possibility that pirenzepine senses small intrinsic differences in receptor structure.

It is evident that pirenzepine is able to discriminate differences in the subclasses of muscarinic binding sites which are not detected by classical antagonists and which make its selective antimuscarinic action possible. While the pattern of selectivity exhibited by pirenzepine favours blockade of the gastric secretagogue action of muscarinic agonists as compared with the effects on the heart, it is possible that drugs will be found with the converse selectivity.

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## (-)-Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor

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The existence of a receptor for  $\gamma$ -aminobutyric acid (GABA) on neurones of the mammalian central nervous system (CNS) is now firmly established<sup>1-3</sup>. It is generally accepted that bicuculline (and its methohalide salts) is an antagonist of the actions of GABA<sup>4,5</sup>, although resistance to bicuculline has been described<sup>6,7</sup>. The view that bicuculline prevents GABA from interacting with a membrane recognition site is supported by results obtained in radiolabelled ligand binding studies<sup>8,9</sup>. Bicuculline-sensitive GABA receptors are not confined to neurones in the CNS but are also present on neurones and axons of the peripheral nervous system<sup>10,11</sup>. Their existence on neurones in sympathetic ganglia made us consider the possibility that they are also present on the terminals of such neurones. This has recently been tested<sup>12,13</sup> by assuming that if GABA depolarises the terminals in a manner similar to the cell bodies, evoked transmitter output might be decreased (see ref. 14). GABA ( $ED_{50}$ , 4  $\mu$ M) clearly decreased the evoked release of accumulated <sup>3</sup>H-noradrenaline from rat atria *in vitro* and <sup>3</sup>H-acetylcholine from preganglionic terminals in the rat superior cervical ganglion *in vitro* without affecting the basal release of tritium. In neither system was the effect of GABA antagonised by bicuculline methobromide, even though the ganglion terminal depolarisation was<sup>13</sup>. This suggested that the two phenomena, depolarisation and inhibition of transmitter release, were separate. The decrease in transmitter release not surprisingly leads to a decrease in the postsynaptic response<sup>15</sup>. Again, the decrease in response was not prevented by bicuculline or other GABA antagonists. We believe that these results indicate the presence of a novel GABA receptor on nerve terminals, a theory supported by results obtained with a variety of GABA analogues. For example, 3-aminopropane sulphonic acid (3-APS) which is at least as active as GABA at bicuculline sensitive sites<sup>10,16,17</sup> is inactive at the terminal receptor. By contrast, the analogue baclofen ( $\beta$ -chlorophenyl GABA) is inactive at bicuculline-sensitive sites<sup>18,19</sup> but is as active as GABA in reducing evoked transmitter output<sup>15</sup>. This effect of baclofen is stereospecific, the (-) isomer being >100-fold more active than the (+) isomer<sup>15</sup>. We now report the presence of the novel GABA receptor within the mammalian CNS.

The technique used in this study was first to allow slices of CNS tissue to accumulate radiolabelled transmitter and then to examine the effect of GABA and its analogues on the basal and K<sup>+</sup>-evoked release into fresh superfusion fluid. Three areas of the rat CNS were chosen, the cerebellum, striatum and frontal cortex. Cerebellar slices were incubated in <sup>3</sup>H-noradrenaline (13 Ci mmol<sup>-1</sup>, Amersham), striatal slices in <sup>3</sup>H-dopamine (24.8 Ci mmol<sup>-1</sup>, NEN) and cortical slices in <sup>3</sup>H-serotonin (29.2 Ci mmol<sup>-1</sup>, NEN). The procedures adopted in each case are given in Figs 1 and 2 legends.

The rate coefficient for basal tritium release from the slices was approximately 0.4% min<sup>-1</sup>. Superfusion of these slices for 2 min with Krebs' solution containing added KCl (final concentration 25 mM) increased the tritium overflow by 100-400% of the basal release. The increase was transient such that the release rate returned to the basal level by the next 4-min collection period. In every experiment the response to K<sup>+</sup> addition declined with repeated applications (Fig. 1b). K<sup>+</sup> additions were therefore limited to three in any one experiment. Control experiments indicated that the increase in tritium overflow in each case was Ca<sup>2+</sup>-dependent. Removal of Ca<sup>2+</sup>

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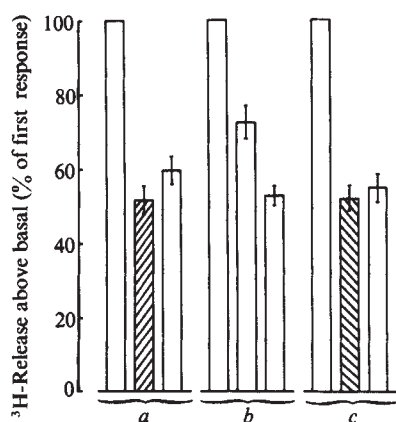
from the superfusion fluid with the addition of EDTA (1 mM) or  $Mg^{2+}$  (2.0 mM) prevented  $K^+$ -induced release.

The effect of GABA and baclofen on  $K^+$ -evoked release of tritiated noradrenaline from cerebellar slices is shown in Fig. 1. Each histogram bar corresponds to the amount of tritium released by 25 mM KCl plotted as a percentage of the initial response. Each panel shows the mean data from five experiments. Slices from the same animal were used in one of the five experiments in each panel. Figure 1b shows the responses to successive additions of KCl. In Fig. 1a and c, ( $\pm$ )baclofen (100  $\mu$ M) and GABA (100  $\mu$ M), respectively, were added 1 min before the second period of superfusion with KCl. In both cases there was ~20% reduction in the expected increase in tritium overflow. When these experiments were repeated with bicuculline methobromide (100  $\mu$ M) present in the superfusion medium, neither the release evoked by  $K^+$  nor the reduction in evoked release produced by GABA and baclofen were affected.

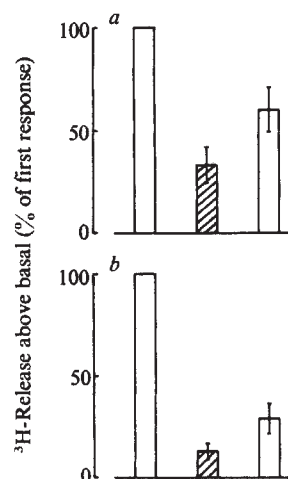
3-APS (100  $\mu$ M) failed to mimic the action of GABA (three experiments). Pretreatment with this GABA analogue did not reduce the  $K^+$ -evoked release whereas GABA (100  $\mu$ M) in the same series of experiments caused the expected reduction. The effect of baclofen was stereospecific; (+)baclofen (100  $\mu$ M) failed to reduce  $^3H$ -noradrenaline release whereas the effect of the (-)isomer was comparable with that of the racemic form.

Similar results were obtained on the evoked release of  $^3H$ -dopamine from striatal slices and  $^3H$ -serotonin from cortical slices (Fig. 2). The output of both of these transmitter substances was depressed by baclofen and GABA although the response to GABA was variable. In the presence of bicuculline, however, only depressant effects were seen. 3-APS was again without depressant activity on transmitter overflow in both systems.

The possibility that GABA affects neurotransmitter release in the mammalian CNS has been previously considered by many



**Fig. 1** Effect of GABA and ( $\pm$ )baclofen on the  $K^+$ -evoked release of  $^3H$ -noradrenaline from rat cerebellar cortex slices. Three or four slices of cortex (0.25 mm thick) obtained from decapitated male rats were incubated for 20 min at 32 °C in 1 ml Krebs' solution containing 0.8  $\mu$ M  $^3H$ -noradrenaline, 0.5 mM iproniazid and 0.1 mM ascorbic acid. The slices were then incubated for a further 20 min in radioactive-free solution at 32 °C before transferring to a superfusion chamber. Oxygenated superfusion fluid was delivered at 0.5 ml min<sup>-1</sup> and 4-min samples collected consecutively. The tritium content of each sample was determined by liquid scintillation spectrometry. Potassium chloride was added (final concentration = 25 mM) to the superfusing fluid for 2-min periods at intervals of 16 min. Each batch of three or four slices was subjected to only three additions of  $K^+$ . The histogram bars represent the increase above basal of the release of tritium in response to  $K^+$  addition. Each has been plotted as a percentage of the initial response. The data are derived from five animals from each of which three batches of slices have been treated separately. Slices from the same animal were used for a control experiment (b, each bar corresponding just to added  $K^+$ ), an experiment where ( $\pm$ )baclofen (100  $\mu$ M) was added 1 min before and during the second period of  $K^+$  addition (a) and an experiment where GABA (100  $\mu$ M) was similarly added (c). Vertical lines on each bar represent s.e.m.



**Fig. 2** Depression of  $K^+$ -evoked release of  $^3H$ -dopamine (a) and  $^3H$ -serotonin (b) by ( $\pm$ )baclofen. Ribbons (0.25 mm  $\times$  0.25 mm) of striatum (a) or frontal cortex (b) were obtained from 200-g male rats. The tissue was incubated for 15 min at 37 °C in 5 ml Krebs' solution containing pargyline (1  $\mu$ M), ascorbic acid (0.2 mM) and either  $^3H$ -dopamine (0.1  $\mu$ M) or  $^3H$ -serotonin (0.1  $\mu$ M). The tissue was then rinsed briefly and aliquots containing approximately 5 mg tissue were transferred to 200- $\mu$ l superfusion chambers. Superfusion at 0.5 ml min<sup>-1</sup> was then begun but the collection of 4-min samples was delayed for a further 30 min. The tritium content of each sample was determined by liquid scintillation spectrometry. In each experiment potassium chloride (final concentration 25 mM) was added to the superfusion fluid on three occasions (for 2-min periods at 12-min intervals). The increase in tritium release provoked by each  $K^+$  addition and represented by histogram bars has been plotted as a percentage of the initial response. The data in a and b are mean values  $\pm$  s.e.m. derived from 16 experiments in each case. ( $\pm$ )Baclofen (100  $\mu$ M) was present 1 min before and during the second period of  $K^+$  addition.

authors. However, conflicting results have been obtained varying from no effect to facilitation of basal or  $K^+$ -induced release<sup>20-30</sup>. These variations may stem from the differences in tissue dimensions used in individual studies or from differences in the duration of superfusion with high  $K^+$  and GABA. A variable which we have considered is the effect of different  $K^+$  concentrations. This we have examined on the cerebellar release of  $^3H$ -noradrenaline. On increasing the  $K^+$  concentration to 35 mM, the reduction in evoked release produced by GABA became more apparent than that at 25 mM. However, at 15 mM  $K^+$ , GABA (100  $\mu$ M) potentiated the evoked release (release in the presence of GABA =  $113 \pm 4.9\%$  of initial response compared with  $80 \pm 11.0\%$  in control experiments; means  $\pm$  s.e.m.,  $n = 6$ ). Potentiation was never obtained with baclofen. Even in the presence of 15 mM  $K^+$  baclofen reduced the evoked release. If the tissue was superfused with bicuculline methobromide (100  $\mu$ M), the potentiation produced by GABA was reversed such that a reduction in evoked release was again observed.

Baclofen, like GABA, is a powerful neuronal depressant in the mammalian CNS and it has been suggested that this effect of baclofen stems from a suppression of transmitter release as well as from a direct postsynaptic action<sup>18</sup>. However, despite its close structural resemblance to GABA, baclofen is generally not considered to be a GABA receptor agonist because of its insensitivity to GABA antagonists<sup>19,31</sup> (but see ref. 18) and inability to displace  $^3H$ -GABA from receptor binding sites<sup>32,33</sup> (but see ref. 34). Our findings suggest that baclofen may mimic the action of GABA at a novel site on nerve terminals. Despite failing to obtain an antagonist for this receptor, our observations<sup>15</sup> of cross-desensitisation, lack of additivity with supramaximal doses and parallelism of log dose-response curves in peripheral models indicate that baclofen and GABA do act at the same site. Potashner<sup>35</sup> and Nistri<sup>36</sup> have previously shown that the evoked release of  $^3H$ -glutamate and acetyl-

choline from guinea pig cortical slices and from hemisected spinal cord, respectively, is reduced by baclofen. It is tempting to believe that their observations result from an action at the same bicuculline-insensitive GABA receptor.

In conclusion, we have shown the existence of a novel GABA receptor which, when activated, decreases transmitter output. As (–)baclofen is the active isomer not only at this receptor<sup>15</sup> but also in producing neuronal depression<sup>37</sup>, its mechanism of action in the therapy of spasticity may indeed be linked with GABA.

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## Compensatory bilateral changes in dopamine turnover after striatal kainate lesion

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Diseases of the extrapyramidal systems like Parkinson's disease and Huntington's chorea have been closely associated with the basal ganglia<sup>1,2</sup>. In particular, the prominent symmetrical nigrostriatal dopaminergic systems play an important role in pathogenesis and symptomatology of motor disorders<sup>3–5</sup>. Biochemical<sup>6,7</sup> and electrophysiological<sup>8,9</sup> data indicate a functional relationship between the nigrostriatal pathways on the two brain hemispheres. Unilateral pharmacological manipulations result in short-term changes in contralateral dopamine (DA) release, and surgical lesions on one side lead to long-term alterations in the firing rate of contralateral dopaminergic neurones. We report here the effects of unilateral kainic acid-induced lesions of striatal cell bodies on ipsi- and contralateral striatal DA

turnover. We present evidence that morphologically intact dopaminergic neurones on both sides possess compensatory mechanisms which can counteract striatal synaptic asymmetries. Such mechanisms could be responsible at least in part for the maintenance of symmetrical motor behaviour in unilaterally lesioned animals.

After anaesthesia with Nembutal (60 mg per kg, intraperitoneally (i.p.)), male Sprague-Dawley rats (200 g) were positioned in a David Kopf small animal stereotaxic apparatus and kainic acid (1 µg in 0.5 µl phosphate-buffered saline (PBS), pH 7.4) was infused unilaterally into the neostriatum (coordinates: 7.9A; 2.6L; 4.8V) as described previously<sup>10</sup>. The animals were killed 2 days to 2 months later by decapitation and the brains rapidly processed according to the Falck-Hillarp technique for the cellular demonstration of catecholamines<sup>11</sup>. The DA stores were measured microfluorimetrically<sup>12</sup>. DA turnover was determined by following DA disappearance after tyrosine hydroxylase inhibition<sup>13</sup>. For this purpose endogenous DA was measured at zero time and at 1 and 2 h after the administration of  $\alpha$ -methyltyrosine methyl ester (H44/68, 250 mg per kg, i.p.). Analysis was performed on injected (kainic acid or PBS), contralateral or intact control striata. The medial parts of the caudate nucleus were assessed individually as described previously<sup>14</sup>. To control for the lesion procedure three kainate-injected rats were killed at 2, 10, 35 and 60 days respectively after the lesion, and their brain processed for morphological analysis by light microscopy.

As confirmed in this study, striatal injections of 1 µg kainic acid induce marked degeneration of neuronal cell bodies throughout the nucleus caudatus, nucleus accumbens and piriform cortex and in parts of the globus pallidus and the part of the neocortex overlying the injected striatum. Also, in accordance with recent immunohistochemical and histological findings, dopaminergic nerve terminals appear to be spared by the lesion with the exception of a small area of nonspecific damage around the needle track<sup>14,15</sup>. No morphological changes could be detected on the contralateral striatum.

Two days after kainic acid injection, DA levels and DA turnover in the medial part of the caudate nucleus were

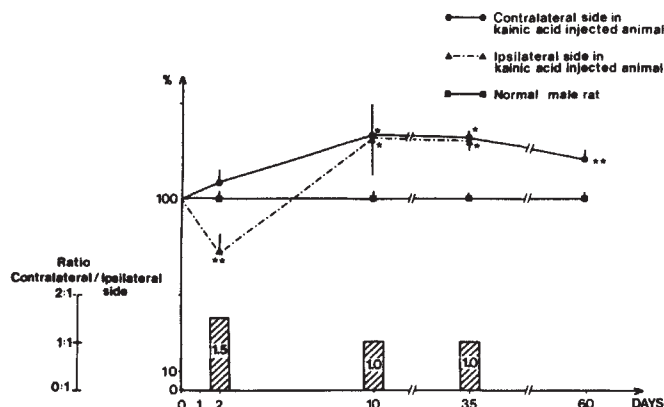
**Table 1** Effects of striatal kainic acid injection on DA levels in medial part of caudate nucleus

| Days after treatment        | Ipsilateral (%) | Contralateral (%) | Normals (%)  |
|-----------------------------|-----------------|-------------------|--------------|
| <i>Kainic acid injected</i> |                 |                   |              |
| 2                           | 130 ± 8* (4)    | 110 ± 6 (4)       | 100 ± 7 (4)  |
| 10                          | 118 ± 9         | 116 ± 9           | 100 ± 3      |
| 35                          | 146 ± 12**†     | 106 ± 4           | 100 ± 3† (4) |
| <i>PBS injected</i>         |                 |                   |              |
| 35                          | 123 ± 4         | 106 ± 2           | 100 ± 3 (5)  |
| <i>Kainic acid injected</i> |                 |                   |              |
| 60                          | —               | 124 ± 4**         | 100 ± 4      |

Kainic acid (1 µg dissolved in 0.5 µl saline) was injected unilaterally in normal male rats 2, 10, 35 or 60 days before killing. PBS (0.5 µl) was injected unilaterally after 35 days. Normal male rats served as controls. Animals were killed by decapitation and the caudate nucleus was taken for histochemical analysis of the DA levels<sup>13</sup>. In the turnover experiments H44/68 (250 mg per kg, i.p.) was injected 1 h or 2 h before decapitation<sup>12</sup>. In some cases at the 35 day time point the entire ipsilateral caudate was taken for biochemical analysis. DA levels are expressed as % of control animals, means ± s.e.m. In the biochemical analysis 100% DA = 8,291 ± 279 ng per mg tissue. The fluorescence values obtained in the quantitative microfluorimetric analysis are given in arbitrary fluorescence units.  $n = 6$  if not otherwise noted within parentheses. Statistical analysis according to Mann-Whitney  $U$ -test (60 day point) or Wilcoxon: one-way classification, treatment versus control (2, 10 and 35 days treatment). \*  $P < 0.05$ ; \*\*  $P < 0.01$ . The measurements were made at around level A8500 according to König and Klippel<sup>29</sup>.

† Biochemical analysis performed on the whole ipsilateral caudate after killing at 35 days.





**Fig. 1** DA turnover changes in ipsi- and contralateral striata at various times after kainate injection. Data represent values in the medial part of the caudate nucleus 2 h after H44/68 treatment. Values below 100% indicate increased DA turnover, and vice versa. Original data are listed in Tables 1 and 2. Vertical bars indicate the ratios of H44/68-induced DA disappearance between contra- and ipsilateral striata. Statistical analysis according to Wilcoxon: one-way classification, treatment versus control. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

increased significantly on the injected side as compared to contralateral or control values (Tables 1 and 2). This increase, synchronous with increased tyrosine hydroxylase activity, increased L-dopa accumulation after dopa-decarboxylase inhibition<sup>10,14</sup> and contralateral turning of the lesioned animal<sup>10</sup>, seems to reflect a functional response of the dopaminergic nerve terminal to an indirect intrastriatal DA release caused by kainic acid<sup>16</sup> and/or to the loss of inhibitory striatonigral feedback mechanisms after degeneration of striatal neurones<sup>17,18</sup>.

By 10 days after the lesion, the increase in DA levels is not as large as 2 days post-injection or has disappeared (Table 1). The DA turnover is now reduced on the lesioned and contralateral side when compared to uninjected control striata (Table 2). The decrements in DA turnover also extend to the central part of the caudate nucleus (level A 8500 according to König and Klippel<sup>29</sup>; L: 2.0 and V extending from +2.0 to 0.0) and to other brain areas rich in dopaminergic nerve terminals like the nucleus accumbens and to a lesser degree the tuberculum olfactorium (data not shown). At 35 days post-lesion significant increases of DA levels were found in the caudatus of the ipsi- and contralateral side; at 60 days significant increases in DA levels were observed in the medial caudate. DA turnover decreases on the contralateral side remain significant at 35 and 60 days after the lesion. At 35 days the biochemical analysis of the entire ipsilateral caudatus still showed reduced DA turnover. Note that injections of PBS-saline into the caudate nucleus did not produce any changes in DA levels and turnover as illustrated in Table 2 for the 35-day post-lesion time point.

At 2 to 3 days after the lesion animals change the direction of spontaneous rotation and start turning towards the lesioned side<sup>10</sup>. Because of the concomitant general hypoactivity of the animals no quantitative evaluation of this turning behaviour (less than one full turn in 5 min) has been performed to date. However, the ipsilateral preference at this stage can be easily observed and the circling strongly accentuated by the administration of dopaminergic drugs<sup>19</sup>. At 5 to 8 days after the lesion the rats stop rotating spontaneously. Figure 1 shows the DA turnover changes at various times after a unilateral striatal kainic acid lesion. Turnover ratios between injected and contralateral striata approach 1.0 at a time when motor asymmetry subsides. Symmetry in striatal DA turnover may therefore contribute to motor symmetry and represent a valid neurochemical marker for the degree of symmetry of motor behaviour in the rat.

The anatomical pathways and biochemical relay systems by which the relevant information is transferred from the lesioned to the contralateral hemisphere are a matter for speculation. Connections within the basal ganglia and bilateral projections

**Table 2** Effects of striatal kainic acid injection on DA turnover in medial part of caudate nucleus

| Days after treatment        |        | Ipsilateral (%)  | Contralateral (%) | Normals (%) |
|-----------------------------|--------|------------------|-------------------|-------------|
| <i>Kainic acid injected</i> |        |                  |                   |             |
| 2                           | H44/68 | 0 h 100 ± 6 (4)  | 100 ± 6 (4)       | 100 ± 7 (4) |
|                             |        | 1 h 54 ± 4** (5) | 77 ± 7 (5)        | 76 ± 3 (5)  |
|                             | H44/68 | 2 h 42 ± 6**     | 63 ± 4            | 58 ± 3      |
| 10                          | H44/68 | 0 h 100 ± 8†     | 100 ± 8†          | 100 ± 3     |
|                             |        | 1 h 92 ± 9*† (5) | 80 ± 10† (5)      | 76 ± 3 (5)  |
|                             | H44/68 | 2 h 77 ± 11*†    | 78 ± 12*†         | 58 ± 3      |
| 35                          | H44/68 | 0 h 100 ± 8      | 100 ± 4           | 100 ± 3 (4) |
|                             |        | 1 h 71 ± 4 (5)   | 80 ± 3 (5)        | 62 ± 3 (4)  |
|                             | H44/68 | 2 h 64 ± 3* (4)  | 78 ± 2* (5)       | 49 ± 2 (4)  |
| <i>PBS injected</i>         |        |                  |                   |             |
| 35                          | H44/68 | 0 h 100 ± 3†     | 100 ± 2           | 100 ± 3 (5) |
|                             |        | 1 h 75 ± 5†      | 75 ± 4            | 72 ± 2 (4)  |
|                             | H44/68 | 2 h 49 ± 4 (4)   | 57 ± 2 (5)        | 59 ± 3 (3)  |
| <i>Kainic acid injected</i> |        |                  |                   |             |
| 60                          | H44/68 | 0 h —            | 100 ± 3           | 100 ± 4     |
|                             |        | 1 h —            | 87 ± 4            | 79 ± 4      |
|                             | H44/68 | 2 h —            | 77 ± 3**          | 64 ± 3      |

Experiments were performed as detailed in Table 1. Values for DA turnover are given as % of levels at zero time. Statistical comparisons were always made with the respective normal group. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

† Biochemical analysis performed on the whole ipsilateral caudate after killing at 35 days.

from the motor cortex to the caudate nucleus may provide the brain with a network of cross-linkages by which fluctuations in one nigrostriatal system may trigger alterations in contralateral DA turnover<sup>20,21</sup>. However, little is known about the biochemical mechanisms associated with this information flow. Dendritic release of DA in the substantia nigra<sup>22-24</sup> may be involved in this process. It has been shown recently that unilateral nigral DA release can inhibit dendritic DA release in the contralateral substantia nigra while simultaneously increasing contralateral striatal DA release<sup>6</sup>.

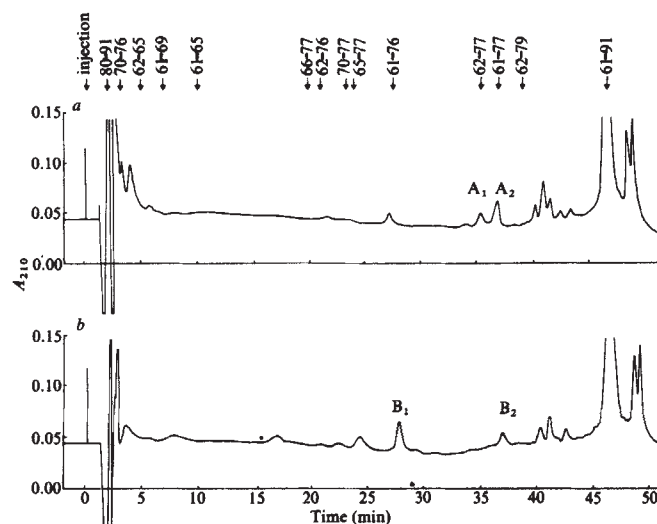
Circling in the rat has commonly been associated with dopaminergic receptor mechanisms<sup>25</sup>. Striatal postsynaptic DA-sensitive adenylate cyclase activity and dopaminergic haloperidol or spiperone binding sites decrease by 85% and 40% respectively, by 2 to 6 days after striatal kainate lesions<sup>26,27</sup>. Receptor loss, which can be demonstrated up to 12 months after kainic acid lesions<sup>28</sup>, is due to a reduction in the number of binding sites rather than to affinity changes and no alterations can be demonstrated on the contralateral side. Post-synaptic parameters therefore do not seem to contribute significantly to the cessation of spontaneous turning behaviour at 5 to 8 days after the lesion. In contrast, our present data indicate that rotation in the rat can be regulated at the pre-synaptic level. It seems likely that compensatory changes in striatal DA turnover represent one of the mechanisms by which rats can maintain symmetrical postures in spite of a drastic unilateral striatal impairment. Clearly this mechanism can only partly explain the return of symmetry, since DA receptor loss exists on the operated side and DA release still occurs on the contralateral side. However, it is conceivable that close scrutiny of origin and regulation of this endogenous adaptational process may contribute significantly to our understanding of human movement disorders.

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**Fig. 1** HPLC fractionation of peptides formed by conversion of  $\beta$ -endorphin by enzymes associated with a SPM preparation. A 10% (w/v) homogenate of rat forebrain in 0.32 M sucrose was centrifuged at  $1,000g_{av}$  for 10 min. The supernatant was centrifuged at  $10,000g_{av}$  for 20 min and the resulting crude mitochondrial-synaptosomal fraction was lysed in distilled water (5 ml per g tissue) at  $0^\circ\text{C}$  for 30 min. The supernatant obtained by centrifugation of the suspension at  $10,000g_{av}$  for 20 min contained the synaptosomal plasma membranes. The membranes were washed and stored at  $-20^\circ\text{C}$ . Incubations were carried out as described in the text. Samples (700–800  $\mu\text{l}$ ) were subjected to HPLC fractionation on a  $\mu$ Bondapak  $C_{18}$  reversed-phase column ( $0.39 \times 30$  cm) eluted with a concave gradient of 10 mM ammonium acetate, pH 4.15 (X) and methanol, containing  $1.5 \text{ ml l}^{-1}$  acetic acid (Y). Initial conditions: X:Y = 70:30, final conditions: X:Y = 25:75. The flow rate was  $2 \text{ ml min}^{-1}$ . HPLC chromatograms of peptide mixtures were obtained after incubation of  $\beta$ -endorphin at a, pH 6.7 and b, pH 5.9. The retention times of a series of reference peptides are indicated at the top. Fractions A<sub>1</sub> and A<sub>2</sub> contained dT $\gamma$ E and  $\gamma$ -endorphin respectively; fractions B<sub>1</sub> and B<sub>2</sub> contained  $\alpha$ -endorphin and  $\gamma$ -endorphin respectively.

## Selective conversion of $\beta$ -endorphin into peptides related to $\gamma$ - and $\alpha$ -endorphin

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**$\beta$ -Endorphin ( $\beta$ -LPH<sub>61-91</sub>)** is a well known endogenous opioid ligand. It and related peptides have recently been implicated in the control of adaptive behaviour. Smaller  $\beta$ -endorphin fragments appeared to be more active moieties than the parent molecule in a number of behavioural situations<sup>1</sup>. Their effects seemed to occur independently of interaction with opiate receptor sites in the brain. Moreover, elimination of the opiate-like properties of  $\gamma$ -endorphin ( $\beta$ -LPH<sub>61-77</sub>) by removing the N-terminal amino acid yielded des-tyrosine- $\gamma$ -endorphin ( $\beta$ -LPH<sub>62-77</sub>, dT $\gamma$ E) which had greater behavioural activity than  $\gamma$ -endorphin<sup>2,3</sup>. The CNS effects of dT $\gamma$ E resembled those of neuroleptic drugs in several test systems.  $\alpha$ -Endorphin ( $\beta$ -LPH<sub>61-76</sub>) exerted effects opposite to those of dT $\gamma$ E and in some aspects its activity was comparable to that of psychostimulant drugs<sup>4</sup>. This opposition of effects suggests that a balance between  $\gamma$ - and  $\alpha$ -type endorphins is involved in the control of brain function<sup>5</sup>. We report here that either  $\gamma$ -endorphin and dT $\gamma$ E or  $\alpha$ -endorphin and des-tyrosine- $\alpha$ -endorphin ( $\beta$ -LPH<sub>62-76</sub>, dT $\alpha$ E) can be formed preferentially from  $\beta$ -endorphin by enzymes associated with an enriched synaptosomal plasma membrane fraction from brain. It is suggested that these enzymes have a role in brain homeostatic mechanisms by regulating the generation of these substances.

Synaptosomal plasma membrane (SPM) preparations from rat brain were obtained by differential centrifugation (see legend to Fig. 1).  $\beta$ -Endorphin (20 nmol) was incubated with the SPM preparation (3.5 mg protein) at  $37^\circ\text{C}$  for 30 min in 1 ml saline buffered with either 25 mM sodium phosphate (pH 7.4, 6.7 and 5.9) or 25 mM sodium acetate (pH 5.0). The reaction was stopped by heating the suspension at  $95^\circ\text{C}$  for 10 min and the membranes were removed by centrifugation. The supernatant

was subjected to high-pressure liquid chromatography (HPLC) fractionation and the peptide-containing fractions were collected. The products were identified by combined application of HPLC and radioimmunoassay (RIA). The HPLC system was able to separate very similar  $\beta$ -endorphin fragments in a highly reproducible manner (Fig. 1). Analysis of their immunoreactivity provided an additional criterion for the identity of peptides cross-reacting in the respective RIA systems.

Briefly, the specificities of the antisera were as follows. For the anti- $\alpha$ -endorphin serum, the percentage cross-reaction compared to  $\alpha$ -endorphin was 110% for dT $\alpha$ E, 2% for  $\gamma$ -endorphin, 5% for dT $\gamma$ E, 1% for  $\beta$ -endorphin and 0.13% for methionine enkephalin. For the anti- $\gamma$ -endorphin serum, dT $\gamma$ E cross-reacted 120%,  $\alpha$ -endorphin 8%, dT $\alpha$ E 0.6%,  $\beta$ -endorphin 4% and methionine enkephalin 0.02%. Details of the RIA systems have been given elsewhere<sup>6</sup>.

$\beta$ -Endorphin was digested by enzymes present in the SPM preparation. HPLC fractionation of the digests revealed the presence of  $\beta$ -endorphin fragments with retention times identical to those of dT $\gamma$ E,  $\gamma$ -endorphin and  $\alpha$ -endorphin in the HPLC system (Fig. 1). Their identity was confirmed by analysis of the immunoreactivity in the appropriate RIA systems (Fig. 2). Essentially the same results were obtained when  $\beta$ -endorphin was incubated with a purified SPM preparation obtained by sucrose density centrifugation as described by Terenius<sup>7</sup>.

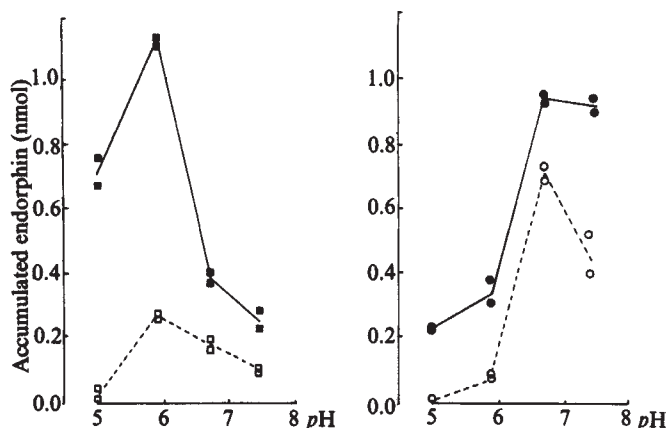
RIA of  $\beta$ -endorphin fragments collected during HPLC fractionation of the digests enabled us to quantitate  $\alpha$ -endorphin, dT $\alpha$ E,  $\gamma$ -endorphin and dT $\gamma$ E. The accumulation of fragments was highly pH-dependent. The largest amounts of  $\alpha$ -endorphin



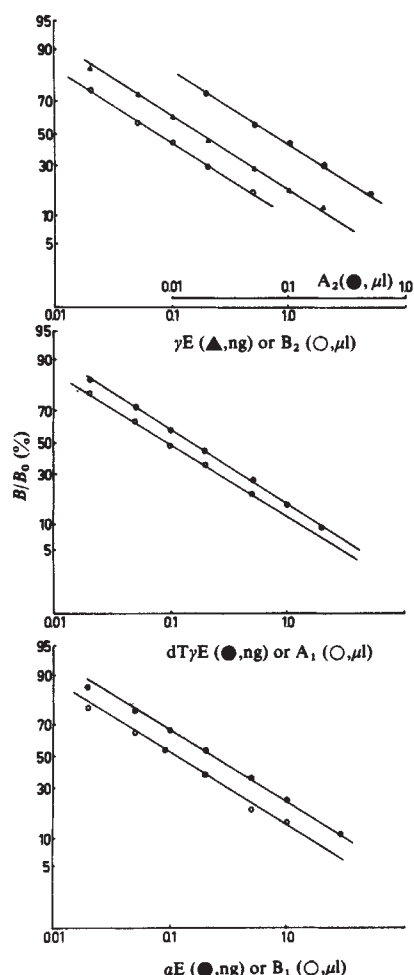
and small amounts of  $\gamma$ -endorphin and dT $\gamma$ E were detected in fractions obtained at pH 5.9. The same pH seemed to be optimal for the accumulation of dT $\alpha$ E, a minor component of the digests. Increasing the pH to 6.7 completely reversed the ratio of  $\alpha$ - to  $\gamma$ -type fragments:  $\alpha$ -endorphin decreased whereas there was a marked increase in both  $\gamma$ -endorphin and dT $\gamma$ E (Fig. 3).

Austen and Smyth, using  $\beta$ -endorphin with the N-terminal tyrosine residue labelled with  $^{125}$ I have demonstrated the production of  $\gamma$ -endorphin at pH 7.4 and of  $\alpha$ -endorphin at pH 5.0 by brain membranes; most of the methionine-enkephalin was also found at pH 5.0 (refs 8, 9). Their experiments were performed in the presence of bacitracin so as to protect the peptide against release of the labelled amino acid residue. The present studies were designed to detect the formation of des-tyrosine endorphins and to extend previous observations to synaptosomal plasma membranes.

Peptide fragments accumulate as a result of both formation and degradation. It has been suggested that initial steps in the degradation of  $\beta$ -endorphin by brain extracts involves cleavages by several endopeptidases, followed by rapid breakdown of the resulting intermediates to free amino acids<sup>10,11</sup>. The present results show that an enriched synaptosomal plasma membrane preparation contains an enzyme system for the conversion of  $\beta$ -endorphin into  $\gamma$ -endorphin, dT $\gamma$ E,  $\alpha$ -endorphin and dT $\alpha$ E. Either dT $\gamma$ E and  $\gamma$ -endorphin or  $\alpha$ -endorphin accumulated preferentially depending on the pH. Thus, the pH appears to determine whether the breakdown of  $\gamma$ -endorphin and dT $\gamma$ E or



**Fig. 3** pH dependence of the accumulation of  $\alpha$ -endorphin (■), dT $\alpha$ E (□),  $\gamma$ -endorphin (●) and dT $\gamma$ E (○) upon incubation of  $\beta$ -endorphin with a SPM preparation. Digests were prepared and fractionated as described in the text and in the legend to Fig. 1. Fractions containing the individual  $\beta$ -endorphin fragments were collected during HPLC fractionation and methanol was removed *in vacuo* at 60 °C. Peptides contained in the residual solution were quantitated in the RIA systems for the various  $\beta$ -endorphin fragments. Values were obtained from two separate experiments.



**Fig. 2** Immunological activity of peptides in fractions A<sub>1</sub>, A<sub>2</sub>, B<sub>1</sub> and B<sub>2</sub> (see Fig. 1 legend) as tested in radioimmunoassay systems for dT $\gamma$ E,  $\gamma$ -endorphin,  $\alpha$ -endorphin and  $\gamma$ -endorphin, respectively. Fractions of the HPLC eluate containing the  $\beta$ -endorphin fragments were collected and methanol was evaporated *in vacuo* at 60 °C. Aliquots of the residual solution (indicated as  $\mu$ l) were subjected to the appropriate RIA. Ordinate shows final fraction bound as a percentage of initial bound.

that of  $\alpha$ -endorphin is the rate-limiting step in the enzymatic conversion of  $\beta$ -endorphin. As a consequence of the mechanism of degradation rather low amounts of the accumulated peptides were detected.

Recently, using HPLC and RIA we have detected des-tyrosine-endorphins in biological material in addition to  $\alpha$ -,  $\gamma$ - and  $\beta$ -endorphin. dT $\gamma$ E and dT $\alpha$ E are present in rat pituitary<sup>6</sup>, in rat brain and in human cerebrospinal fluid (J. P. H. B. *et al.*, in preparation). Together with the present study this observation supports our view that des-tyrosine-endorphins are endogenous peptides and are formed by selective cleavage of  $\beta$ -endorphin.

$\beta$ -Endorphin fragments may be factors involved in behavioural adaption. According to the hypothesis put forward by De Wied<sup>5</sup>, normal brain functioning would require a balance between dT $\gamma$ E or a peptide related to it and  $\alpha$ -type endorphins. Consequently, the enzyme system acting in our experiments may provide a regulatory mechanism for the turnover of the two ligands. The preferential formation of dT $\gamma$ E and  $\gamma$ -endorphin or  $\alpha$ -endorphin over a narrow, near physiological pH range shows that the enzyme system can be modulated. The experimental pH changes may have mimicked physiological processes leading to this modulation. Thus, by showing the formation of dT $\gamma$ E,  $\gamma$ -endorphin and  $\alpha$ -endorphin by enzymes present in an enriched synaptosomal plasma membrane fraction, the experiments described here may have provided a biochemical basis for the hormonal regulation of adaptive behaviour.

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## Parallel pathways for transduction of chemotactic signals in *Escherichia coli*

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Bacteria respond transiently to tactic stimuli<sup>1</sup>, which indicates that cells adapt to new levels of stimulation. Receptors<sup>2,3</sup> send signals to a tumble regulator that controls the balance between directions of flagellar rotation<sup>4</sup>, creating a pattern of swimming and tumbling that results in taxis<sup>5</sup>. In *Escherichia coli*, mutations in *tsr* and *tar* affect receptor-regulator linkages, eliminating responses mediated by two different groups of receptors<sup>6-9</sup>. Sensitivity to most sugars is not reduced by either mutation but observations that *tsr-tar* double mutants do not respond to spatial gradients of any compound led to the suggestion that the *tsr* and *tar* products together provide a necessary final step in transduction from all receptors<sup>7,8</sup>. Another class of pleiotropic taxis mutations, *trg*<sup>10-13</sup>, causes defective transduction from only two receptors, those for galactose and ribose. We were therefore interested in determining whether the *trg* function represented an early stage of transduction or whether it was analogous to the *tsr* and *tar* functions. We have examined the sensitivity of *tsr* and *tar* double mutants to temporal gradients, prompted by a report that such gradients of galactose or ribose evoke responses in those strains<sup>14</sup>. We report here that *tsr-tar* double mutants were sensitive to stimulation by compounds to which both single mutants respond but were drastically defective in adaptation to these stimuli. We suggest there are at least four parallel, single-component pathways for transduction of tactic signals, from the Tsr, Tar, Trg and enzyme II groups of receptors.

We used four *tsr-tar* double mutants (see Table 1 legend) and confirmed that they tumble very infrequently<sup>7-9</sup>. *tsr-tar* cells tethered to a glass surface spun only in the counterclockwise, smooth-swimming direction, in contrast to wild-type cells which frequently reverse their direction of rotation. However, undisturbed cells initiated reversals at times ranging from 5 to 40 min after tethering and subsequently behaved much like wild-type cells. We believe the delayed onset of a normal rotational pattern results from the adaptational defect of these mutants documented below. Lack of tumbling by swimming cells and absence of clockwise rotation by tethered cells would then be a reflection of extended times necessary for adaptation to stimuli received during prior manipulation (such as changes in temperature or concentration of O<sub>2</sub> or K<sup>+</sup> ion).

After tethered *tsr-tar* cells had begun to exhibit normal rotational patterns, we applied maximal temporal stimuli, gradients from 0 to 10 mM, of the compounds listed in Table 1. Compounds inactive in either *tsr* or *tar* mutants were predictably inactive in the double mutants. Two amino acids, asparagine and cysteine, which evoke significant responses in both *tsr* and *tar* mutants<sup>6</sup>, did not stimulate a double mutant. This is consistent with the notion that receptors for these compounds interact with both the *tsr* and *tar* transductional pathways and one or the other must be functional for a response<sup>7,8</sup>. In contrast, *tsr-tar* double mutants responded to sugars in the same way as wild-type cells, by complete suppression of tumble-mode rotation. However, adaptation was significantly delayed after maximal stimuli of sugars recognised by enzyme II receptors<sup>3,15</sup> and did not occur even after prolonged times for those recognised by *trg*-related receptors. Delayed adaptation did occur after moderate stimuli of allose and fucose, non-metabolisable analogues of ribose and galactose, respectively (Fig. 1). Behaviour of a *tsr-tar* double mutant lacking the galactose receptor as the result of an *mgIB* muta-

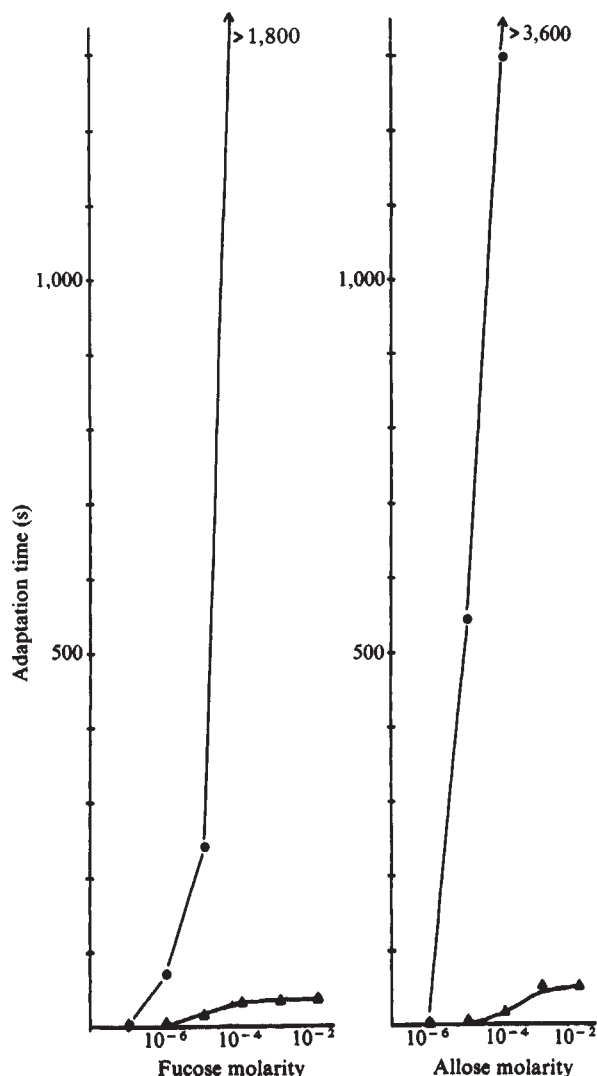


Fig. 1 Adaptation times as a function of temporal gradient. Cells were grown on salts plus ribose for allose stimulation or plus glycerol and 1 mM fucose for fucose stimulation were tethered as for Table 1 and submitted to temporal gradients from 0 to the concentration on the abscissa. For the wild-type RP437 (▲), maximal response to fucose gradients was 35 s and to allose gradients 50 s. Half-maximal times corresponded to 13 and 220  $\mu$ M, respectively. The fucose-stimulated *tsr-tar* strain was RP4375 and the allose-stimulated, RP4372 (both ●).

tion<sup>16</sup> (Table 1) demonstrates that responses observed in *tsr-tar* double mutants require the appropriate chemoreceptor. In addition, it eliminates the possibility that responses to enzyme II sugars are caused solely by low level contamination with galactose or glucose, which are recognised at micromolar concentrations by the galactose receptor.

When an attractant recognised by an enzyme II receptor was washed away from adapted *tsr-tar* cells, reversals between clockwise and counterclockwise rotation ensued at high frequency for periods of the order of a minute. Wild-type cells stimulated by 10 mM to 0 gradients of those sugars exhibit only a few seconds of clockwise rotation. Thus, it seems that *tsr-tar* double mutants are also sensitive to unfavourable stimuli mediated by sugar receptors and, as in the case of favourable stimuli, are defective in adaptation.

A *trg* null mutation<sup>12</sup> was introduced into the four *tsr-tar* double mutants. We found that tethered cells of *tsr-tar-trg* triple mutants rotated exclusively in the smooth-swimming direction and, in contrast to the double mutants, never showed a relaxation to normal alternating rotational behaviour. We believe this phenotype to be a more drastic extension of the adaptational defect of the double mutants, implying that the *trg* product is involved in adaptation as well as transduction.



**Table 1** Adaptation times of tethered cells to maximal temporal stimuli

| Attractant                  | Receptor                | Transducer | Adaptation time (s) |                |                |                     | Ratio<br><i>tsr tar</i> /WT |
|-----------------------------|-------------------------|------------|---------------------|----------------|----------------|---------------------|-----------------------------|
|                             |                         |            | WT                  | WT <i>mgIB</i> | <i>tsr tar</i> | <i>tsr tar mgIB</i> |                             |
| Galactose                   | GBP                     | Trg        | 30                  | ND             | > 1,800        | ND                  | > 60                        |
| Fucose                      | GBP                     | Trg        | 33                  | 0              | > 1,800        | 9                   | > 55                        |
| Ribose                      | RBP                     | Trg        | 50                  | ND             | > 1,800        | ND                  | > 36                        |
| Allose                      | RBP                     | Trg        | 48                  | 50             | > 1,800        | > 1,800             | > 37                        |
| Glucose*                    | enz II <sup>glu</sup> * | Unident.   | ND                  | 52             | > 1,800        | 157                 | 3                           |
| Fructose                    | enz II <sup>fru</sup>   | Unident.   | 41                  | 32             | 540            | 390                 | 12                          |
| Sorbitol                    | enz II <sup>abl</sup>   | Unident.   | 55                  | 15             | 642            | 353                 | 24                          |
| <i>N</i> -acetylglucosamine | enz II <sup>NAG</sup>   | Unident.   | 72                  | 55             | 480            | 239                 | 4                           |
| Aspartate                   | Unident.                | Tar        | 258                 | ND             | 60             | 0                   | 0                           |
| $\alpha$ -methylaspartate   | Unident.                | Tar        | 221                 | ND             | 26             | 0                   | 0                           |
| Serine                      | Unident. (2)            | Tsr + Tar  | 790                 | 720            | 49             | 18                  | 0.02                        |
| Asparagine                  | Unident.                | Tsr + Tar  | ND                  | 258            | ND             | 0                   | 0                           |
| Cysteine                    | Unident.                | Tsr + Tar  | ND                  | 330            | ND             | 0                   | 0                           |

Cells were grown at 37 °C in minimal salts<sup>10</sup> plus ribose (*mgIB*) or ribose and 1 mM fucose (*mgIB*<sup>+</sup>). No special measures were taken to induce maximal levels of enzymes II. Flagella were sheared, cells tethered by anti-flagella serum, stimulated by temporal gradients at 37 °C and observed on videotape recordings as described elsewhere<sup>11</sup>. Adaptation time for a given cell was the time after stimulation by an attractant when the first complete clockwise rotation occurred. Values given are mean times for 10–20 cells in one or usually two experiments. Standard deviations were 20–30% for short (~60 s) times and somewhat less for longer times. *tsr-tar* double mutants often exhibited poor motility and thus could be tethered without shearing. Such cells initiated normal rotational behaviour (see text) sooner than sheared cells. A change of buffer surrounding tethered cells results in an inhibition of clockwise rotation for an average of 5–10 s in wild-type cells and 30–150 s in *tsr-tar* cells, the values varying for different preparations of tethered cells. These background responses have been subtracted from the raw data to yield the values given. As in the case of *cheX* mutants<sup>19</sup>, the double mutants had a tendency to lose the ability to rotate clockwise after extended times (> 1 h) in chemotaxis buffer. The wild-type (WT) strain was RP437 (ref. 21) and the *tsr-tar* mutant RP4375 (*tsr* 2-3-4 *tar* 52Δ1). Similar behaviour was observed for RP4372 (*tsr* 518 *tar* 52Δ1), RP4373 (*tsr* 518 *tar* 1) and RP 4376 (*tsr* 2-3-4 *tar* 1). An *mgIB::Tn5* mutation (S. Harayama and G.L.H., unpublished) was introduced by transduction, and the absence of the gene product confirmed by SDS-polyacrylamide gel electrophoresis<sup>22</sup>. *trg2::Tn10* (ref. 12) was introduced into each *tsr-tar* double mutant by transduction and the presence of the mutation confirmed by transduction of the Tc<sup>r</sup>, Trg character back into a wild-type strain. All compounds were obtained from commercial sources and used without further purification. Ratios of mutant to WT response were calculated using values for *mgIB* derivatives where adaptation times of those derivatives were shorter than the parent strain, indicating that the sugar might contain contaminating glucose or galactose. GBP, galactose-glucose-binding protein; RBP, ribose-binding protein; MBP, maltose-binding protein; Unident., unidentified; ND, not determined.

\* Glucose is recognised by three receptors, GBP, enz II<sup>glu</sup> and enz II<sup>man</sup>. There is very little enz II<sup>man</sup> receptor activity in these strains.

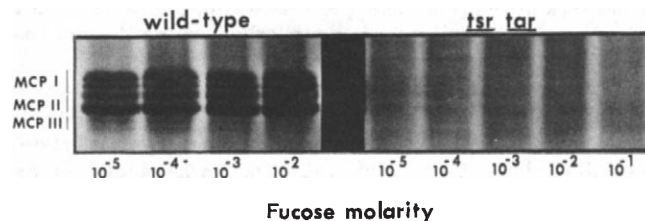
The relationship between transduction and adaptation merits further discussion. The *tsr* and *tar* products, phenotypically identified as transducers, are directly involved in adaptation to the stimuli they transduce<sup>14</sup>. Adaptation is correlated with carboxymethylation or demethylation of the two products, termed methyl-accepting protein (MCP) I and II, respectively<sup>7,8</sup>. Recently, an independent MCP III was shown to be methylated on stimulation by compounds recognised by galactose and ribose receptors<sup>13</sup>. MCP III is not methylated in *trg* mutants and may well be the *trg* product. These observations correlate well with our conclusions that *trg* represents an independent transducer that is also involved in adaptation.

*tsr-tar* double mutants lack only two transducer/MCPs and exhibit correspondingly limited defects in tactic sensitivity. However, the mutants are generally defective in adaptation. Interestingly, *tsr* and *tar* single mutants swim smoothly for longer times than wild-type cells after temporal stimulation by active compounds<sup>7</sup>. The extended times were considered to be enhanced responses<sup>7</sup>; we suggest instead that they indicate moderately defective adaptation. Modest extensions of adaptation times may result in increased accumulations in spatial gradients<sup>6,7</sup>, but mutants exhibiting very long adaptation times do not accumulate at all (ref. 7 and the present study). It is striking that *tsr-tar* mutants are as defective in adaptation to maximal stimuli of *trg*-related compounds as are cells unable to methylate because of methionine starvation<sup>17</sup> or a defective methyltransferase<sup>18,19</sup>, even though methionine, the transferase and the appropriate transducer/MCP are present.

The extended adaptation times of *tsr-tar* double mutants pose the question of whether methylation levels are correspondingly increased, as might be expected from the correlation of methylation increases and adaptation in wild-type cells<sup>20</sup>. This does not seem to be the case. Double mutants stimulated by gradients of fucose (Fig. 2) or allose (data not shown) exhibit very little detectable methylation, whether the stimulus is one to which the

mutant adapts after extended times or one to which it does not adapt in the time of the experiment (see Fig. 1). Basal levels of MCP III methylation in *tsr-tar* mutants are low<sup>13</sup> and adaptation by wild-type strains to maximal *trg*-transduced stimuli corresponds to a 50% increase in MCP III methylation. Thus, it is not possible to conclude from Fig. 2 whether both adaptation and MCP III methylation simply proceed slowly in *tsr-tar* mutants or whether adaptation occurs slowly by a process independent of methylation.

The observation that *tsr-tar* double mutants respond to temporal gradients of sugars demonstrates that the *tsr* and *tar* products together do not transduce signals from all receptors<sup>7-9</sup>. Identification of defective adaptation rather than defective response as the basis for the inability of double mutants to accumulate in spatial gradients of sugars removes the remaining



**Fig. 2** Methylation patterns of wild-type cells and a *tsr-tar* mutant after stimulation by fucose. Cells were grown in salts plus ribose and 1 mM fucose, washed, resuspended, exposed to [<sup>3</sup>H] methionine (12 Ci mmol<sup>-1</sup>, Amersham), subjected to temporal gradients from 0 to the concentration indicated and collected 30 min after stimulation essentially as described previously<sup>7</sup>. Approximately 10<sup>8</sup> cells were subjected to electrophoresis on an SDS-10% polyacrylamide gel. The gel was prepared for fluorography and exposed to X-ray film<sup>23</sup>. Only the section of the autoradiogram including the area of known MCPs (apparent molecular weights 56,000–65,000 (ref. 7)) is shown. Wild-type and *tsr-tar* are as in Table 1. The range of methylated bands that constitute MCPs I and II are indicated based on the scheme in ref. 7. We have observed that MCP III includes at least two methylated bands (P.E. and G.L.H., in preparation).

barrier to postulating that the *tsr*, *tar* and *trg* products function as analogous, independent transducers. All data now suggest that the link between any receptor and the tumble regulator is a single transducer protein and that the protein is an MCP, methylated during adaptation to the stimuli it transduces. No observations remain that require postulation of multi-step linkages between receptor and regulator<sup>1,3,13,14</sup>.

We suggest that there is at least one additional transducer/MCP, serving enzyme II receptors, as *tsr*, *tar* or *trg* mutations do not eliminate responses to enzyme II sugars<sup>9,11,13</sup>. Also, *trg*-transduced stimuli can be distinguished from enzyme II-mediated stimuli by the inability of *tsr-tar* double mutants to adapt to maximal levels of *trg* stimuli (Table 1).

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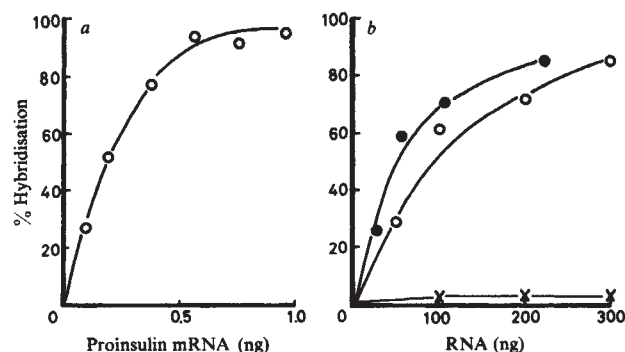
## Translational control of proinsulin synthesis by glucose

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Glucose is known to stimulate proinsulin synthesis in pancreatic islets of rats as well as of many other animals<sup>1</sup>, but the mechanism involved remains uncertain. If proinsulin induction by glucose is regulated at the transcriptional level, the amount of proinsulin mRNA may be increased by the administration of glucose. Alternatively, if proinsulin induction is regulated at the translational level, the rate of proinsulin synthesis may not be correlated with the amount of proinsulin mRNA. We have now used a proinsulin cDNA hybridisation method to determine the amount of proinsulin mRNA and the subcellular distribution of proinsulin mRNA in rat pancreatic islets. The results suggest that glucose-induced proinsulin synthesis is mainly achieved by enhancing the translation efficiency of proinsulin mRNA pre-existent on the membrane-bound polysome in pancreatic islets.

Proinsulin mRNA was purified from rat B-cell tumours induced with streptozotocin and nicotinamide, and proinsulin cDNA was synthesised using purified proinsulin mRNA as a template<sup>2–4</sup>. The fidelity of the hybridisation reaction with proinsulin mRNA and its cDNA was assessed from the follow-



**Fig. 1** Quantification of proinsulin mRNA by hybridisation with proinsulin cDNA. <sup>3</sup>H-proinsulin cDNA was synthesised using proinsulin mRNA purified from rat B-cell tumours as a template as described previously<sup>3,4</sup>. The hybridisation reaction was carried out at 65 °C for 20 h in a 50-μl reaction mixture (0.5 M NaCl, 25 mM HEPES-NaOH, pH 7.3, 1 mM EDTA, 0.1% SDS, 4 μg yeast RNA) after heating at 100 °C for 2 min. The reaction was stopped by adding 0.4 ml of 0.2 M sodium acetate (pH 4.5) containing 0.4 M NaCl, 2 mM zinc acetate and 6,000 units of nuclease S<sub>1</sub> (Seikagaku Kogyo), followed by incubation at 37 °C for 1.5 h. After incubation, 200 μg yeast DNA was added to the incubated mixture, and the nuclease S<sub>1</sub>-resistant hybrid was precipitated with cold 10% trichloroacetic acid, and collected on a Toyo membrane filter TM-2. The filter was dissolved in 2 ml Cellosolve and the radioactivity was counted in a toluene-based scintillation solution. Total RNA was prepared from rat pancreatic islets, rat B-cell tumours and rat liver with the phenol extraction and LiCl-precipitation procedures<sup>3</sup>. *a*, Hybridisation of purified proinsulin mRNA with <sup>3</sup>H-proinsulin cDNA. *b*, Hybridisation of the total RNA from pancreatic islets (●), B-cell tumours (○) and liver (×) with <sup>3</sup>H-proinsulin cDNA.

ing evidence: (1) the hybridisation occurred within two orders of magnitude, as expected of a pseudo-first order reaction<sup>3</sup>; (2) the experimental  $R_{0t_{1/2}}$  value ( $2.1 \times 10^{-4}$ ) was in good agreement with the expected value ( $2.7 \times 10^{-4}$ ) calculated from the  $R_{0t_{1/2}}$  value of the hybridisation with mouse globin mRNA and its cDNA<sup>3</sup>; (3) a sharp thermal denaturation curve of the hybrid with  $T_m$  of 74 °C was obtained (data not shown). As shown in Fig. 1a, trace amounts of proinsulin mRNA (0.05–0.5 ng) could be measured by hybridisation with proinsulin cDNA. As it is difficult to prepare many islets from rat pancreas, this sensitive hybridisation assay is of particular value. To examine the specificity of the hybridisation reaction, we prepared total RNA from rat pancreatic islets, rat B-cell tumours and rat liver, and examined the hybridisation of proinsulin cDNA with total RNA from these tissues (Fig. 1b). From the extent of the hybridisation, the proinsulin mRNA content of total RNA from islets and that from B-cell tumours were determined to be 0.38% and 0.25%, respectively. As the proinsulin mRNA content of total RNA from islets and B-cell tumours determined by the hybridisation method was consistent with that determined by a wheat-germ cell-free translation assay<sup>3–5</sup>, it is reasonable to assume that almost all the amount of proinsulin mRNA determined by the hybridisation method was translatable proinsulin mRNA. Total RNA of rat liver did not hybridise with proinsulin cDNA. These results suggest that the hybridisation reaction was highly specific.

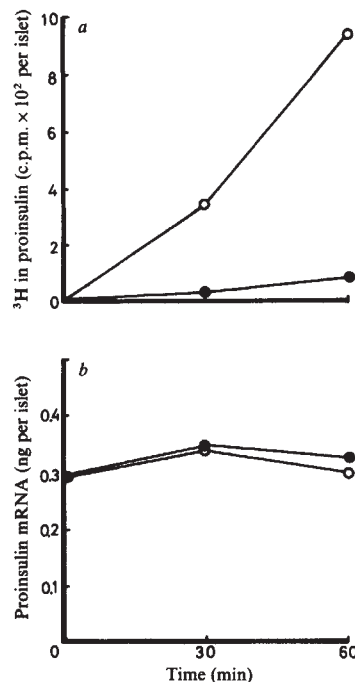
The effects of glucose on proinsulin synthesis in isolated pancreatic islets of rats were examined. When the concentration of glucose in the incubation medium was increased from 2.8 mM to 25 mM, the proinsulin synthesis was greatly stimulated (~10-fold) (Fig. 2a) and proinsulin synthesis in the glucose-stimulated islets comprised 53% of the total protein synthesis. In contrast, the non-proinsulin protein synthesis was only slightly stimulated (~1.3-fold). These results suggest that the glucose-stimulation effect on protein synthesis in islets is highly specific to proinsulin synthesis.

In a wheat-germ cell-free system, we found that the amount of proinsulin mRNA in islets as determined by translation assay

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**Fig. 2 a**, Time course of induction of proinsulin synthesis by glucose in pancreatic islets. Pancreatic islets were prepared from male Wistar rats (200–240 g), which had been fasted for 18 h, in mild conditions of collagenase treatment as described previously<sup>6</sup>. Twenty islets were incubated at 37 °C for the indicated time in 125  $\mu$ l Dulbecco's modified Eagle's medium (without glucose and leucine) containing 10  $\mu$ Ci <sup>3</sup>H-leucine (57.4 Ci mmol<sup>-1</sup>, NEN), 2.8 mM (●) or 25 mM (○) glucose and 20% fetal calf serum (GIBCO). After incubation, the incubated islets were washed three times with 1 ml ice-cold Hank's solution, suspended in 0.2 ml of 60 mM Tris-phosphoric acid (pH 6.8) containing 1% SDS, 5% 2-mercaptoethanol and 10% glycerol, and sonicated for 10 s with a microtip of a Kontes ultrasonic cell disruptor. The sonicated material was heated at 100 °C for 2 min. An 80- $\mu$ l aliquot of the heated sample was submitted to SDS-polyacrylamide (15%) gel electrophoresis (1  $\times$  4 cm)<sup>7</sup>. After electrophoresis, the gel was cut into 1-mm slices and extracted with a Gilson B-100 gel fractionator. The radioactivity of the gel slice was counted in a toluene-based scintillation solution. The amount of newly synthesised proinsulin was calculated by summing <sup>3</sup>H-leucine radioactivity of the gel slices corresponding to the proinsulin peak<sup>4,5</sup>. **b**, Time course of proinsulin mRNA level during induction of proinsulin synthesis by glucose in pancreatic islets. Twenty-five islets were incubated at 37 °C for the indicated time in the presence of 2.8 mM (●) or 25 mM (○) glucose as described in Fig. 2a, except for addition of 0.2 nmol unlabelled leucine instead of 10  $\mu$ Ci <sup>3</sup>H-leucine. The incubated islets were washed once with 1 ml ice-cold Hank's solution and sonicated for 20 s with a microtip of a Kontes ultrasonic cell disruptor in 0.5 ml of 0.1 M Tris-HCl (pH 9) containing 10 mM EDTA, 0.1 M dithiothreitol, 2% SDS and 20  $\mu$ g yeast RNA and an equal volume of a phenol solution (phenol/chloroform/isoamylalcohol 50:50:2) equilibrated with 0.1 M Tris-HCl (pH 9) containing 10 mM EDTA. The emulsion was vigorously shaken at room temperature for 5 min, left on ice for 5 min and centrifuged to separate two phases. The water phase was removed, and the phenol phase was re-extracted with 0.5 ml of 0.1 M Tris-HCl (pH 9) containing 10 mM EDTA and 2% SDS. The combined water phases were re-extracted with the phenol solution. Nucleic acid was precipitated by adding 0.1 vol. of 2 M sodium acetate (pH 5.5) plus 2.5 vol. of ethanol at -20 °C for 16 h. The precipitate was collected by centrifugation and washed once with 2 ml of cold 75% ethanol. Aliquots of the nucleic acid were used for quantification of proinsulin mRNA by hybridisation with proinsulin cDNA.



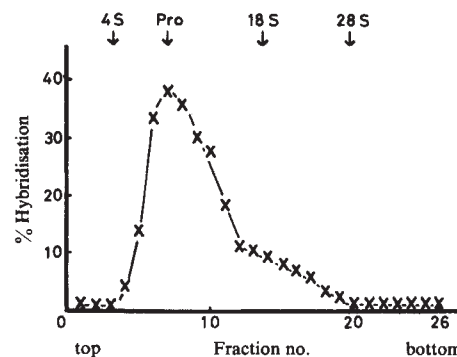
remained unchanged during the period of proinsulin induction by glucose for up to 60 min<sup>4,5</sup>. In the present paper, we have confirmed our previous result by the hybridisation method. The amount of proinsulin mRNA in the glucose-stimulated (25 mM glucose) or -unstimulated (2.8 mM glucose) islets remained unchanged during the period of proinsulin induction for up to 60 min (Fig. 2b).

To examine the subcellular localisation of proinsulin mRNA in the glucose-stimulated or -unstimulated islets, we prepared subcellular fractions from the islets. After pancreatic islets were incubated in a medium containing 2.8 mM or 25 mM glucose for 60 min at 37 °C, the islets were homogenised with rat liver as carrier, and membrane-bound polysome, free polysome and post-polysomal supernatant fractions were prepared. RNA was extracted from the subcellular fractions. Aliquots of the extracted RNA were used for quantification of proinsulin mRNA (Table 1). Recoveries of proinsulin mRNA in the membrane-bound polysome fractions were 58% for glucose-stimulated and 40% for unstimulated islets. However, a significant amount of proinsulin mRNA was also detected in the post-polysomal supernatant fraction; this was more marked in the unstimulated islets.

In some eukaryotic cells, some mRNAs have been shown to be present in a post-polysomal supernatant fraction as messenger ribonucleoprotein (mRNP; actin mRNP<sup>9</sup>, histone mRNP<sup>10</sup>, globin mRNP<sup>11</sup>, albumin mRNP<sup>12</sup>). As it has been suggested that the mRNP may be involved in the regulation of the mRNA translation<sup>9,12</sup>, it would be interesting to determine whether proinsulin mRNA is present in the post-polysomal supernatant fraction complexed with protein to form mRNP. To characterise the form of proinsulin mRNA present in the supernatant fraction, the supernatant was fractionated by sucrose gradient centrifugation and proinsulin mRNA sequences in each gradient fraction were quantified with proinsulin cDNA. The sedimentation profile of proinsulin mRNA sequences in the supernatant fraction exhibited an almost single major peak in the region of approximately 9S, which corresponds to the sedimentation value of intact proinsulin mRNA

free from protein (Fig. 3)<sup>3</sup>. This suggests that proinsulin mRNA detected in the post-polysomal supernatant exists as an intact and free form.

The present experiment with isolated pancreatic islets of rats shows that the amount of proinsulin mRNA remains unchanged during the induction of proinsulin synthesis by glucose. Thus glucose regulates proinsulin synthesis at the translation level (translational control). Proinsulin mRNA, and mRNAs coding for other secretory proteins, have been shown to be translated on the membrane-bound polysome<sup>13</sup>. From the subcellular distribution of proinsulin mRNA in the glucose-stimulated or



**Fig. 3** Sucrose gradient sedimentation profile of proinsulin mRNA sequences in post-polysomal supernatant. After concentration with a Toyo membrane filter UK-50, an aliquot of the post-polysomal supernatant fraction of glucose-unstimulated islets was applied on a linear 15–30% sucrose gradient made in 10 mM Tris-HCl (pH 7.6) containing 0.5 M KCl, 2 mM EDTA and 100  $\mu$ g ml<sup>-1</sup> heparin. Centrifugation was performed at 60,000 r.p.m. at 0 °C for 3.5 h in a Beckman SW 60 Ti rotor. The gradient was fractionated with a Buchler Instrument Densi-Flow. From each fraction, RNA was extracted and used for quantification of proinsulin mRNA by hybridisation with proinsulin cDNA. Arrows indicated relative sedimentation of: 4S, rat liver tRNA; Pro, proinsulin mRNA; 18S, rat liver 18S rRNA; 28S, rat liver 28S rRNA.

**Table 1** Subcellular distribution of proinsulin mRNA in pancreatic islets

| Glucose | Membrane-bound polysome | Free polysome | Post-polysomal supernatant |
|---------|-------------------------|---------------|----------------------------|
| Expt 1  |                         |               |                            |
| 2.8 mM  | 0.099 (39.4)            | 0.033 (13.1)  | 0.119 (47.5)               |
| 25 mM   | 0.136 (57.9)            | 0.028 (11.9)  | 0.071 (30.2)               |
| Expt 2  |                         |               |                            |
| 2.8 mM  | 0.109 (41.4)            | 0.031 (11.8)  | 0.123 (46.8)               |
| 25 mM   | 0.145 (58.4)            | 0.036 (14.5)  | 0.067 (27.1)               |

One hundred and fifty islets were incubated at 37 °C for 60 min in the presence of 2.8 mM or 25 mM glucose as described in Fig. 2a, except for addition of 0.2 nmol unlabelled leucine instead of 10  $\mu$ Ci  $^3$ H-leucine. The incubated islets were washed once with 1 ml ice-cold Hank's solution, added to 0.1 g wet weight of rat liver as carrier and homogenised at 0 °C with 10 strokes of a motor-driven glass-Teflon Potter-Elvehjem homogeniser in 3.9 ml of 250 mM sucrose containing 10 mM HEPES-KOH (pH 7.4), 75 mM KCl, 5 mM MgCl<sub>2</sub>, 3 mM glutathione and 0.5% diethyl pyrocarbonate. The homogenate was centrifuged at 2,000 r.p.m. at 0 °C for 10 min in a Sorvall HB-4 rotor. After addition of heparin (100  $\mu$ g ml<sup>-1</sup>), membrane-bound polysome, free polysome and post-polysomal supernatant were prepared from the post-nuclear supernatant essentially according to the same methods as of Ramsey and Steele<sup>8</sup> and Bag and Sarkar<sup>9</sup>. Each subcellular fraction was precipitated with cold 10% trichloroacetic acid and washed with cold 75% ethanol and diethyl ether. Nucleic acid was extracted from the trichloroacetic acid-precipitate as described in Fig. 2b. Aliquots of the nucleic acids were used for quantification of proinsulin mRNA by hybridisation with proinsulin cDNA. Values are expressed as ng proinsulin mRNA per islet. The numbers in parentheses give the percentage of the post-nuclear supernatant.

-unstimulated islets, we can speculate as to possible mechanisms of translational control. Although the rate of proinsulin synthesis in the glucose-stimulated islets was 10 times that in the unstimulated islets, only a small difference between the amount of proinsulin mRNA in both membrane-bound polysome fractions was observed (58% and 40% in the stimulated and unstimulated islets, respectively). These results indicate that proinsulin synthesis is mainly regulated by enhancing the translation efficiency of proinsulin mRNA on the membrane-bound polysome.

A significant amount of proinsulin mRNA was also detected in the post-polysomal supernatant fraction (Table 1). The amount of proinsulin mRNA in the supernatant fraction was decreased by glucose stimulation. This decrease in proinsulin mRNA in the supernatant fraction was closely correlated with an increase in proinsulin mRNA in the membrane-bound polysome fraction. Therefore, the regulation by glucose of proinsulin synthesis in pancreatic islets is assumed to be achieved also in part by the transfer of proinsulin mRNA in the supernatant to the membrane-bound polysome.

Recent observations indicate that the rates of synthesis in proteins such as ovalbumin<sup>14</sup>, globin<sup>15</sup>, immunoglobulin<sup>16</sup>, growth hormone<sup>17</sup>, tyrosine aminotransferase<sup>18</sup> and tryptophan oxygenase<sup>19</sup> are closely correlated with the amount of the corresponding mRNA in cells. Therefore, the expression of genes of these proteins as well as many other proteins in eukaryotic cells is believed to be regulated at the transcription level.

The translational control of proinsulin synthesis by glucose as reported here seems to be a unique regulation of protein synthesis in eukaryotic cells. As insulin regulates the glucose level in the blood, the rates of both secretion and synthesis of insulin are required to change rapidly according to the change of the blood glucose level. Translational control may be a means of ensuring rapid regulation of proinsulin synthesis by glucose.

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## A $\rho$ -recognition site on phage $\lambda$ *cro*-gene mRNA

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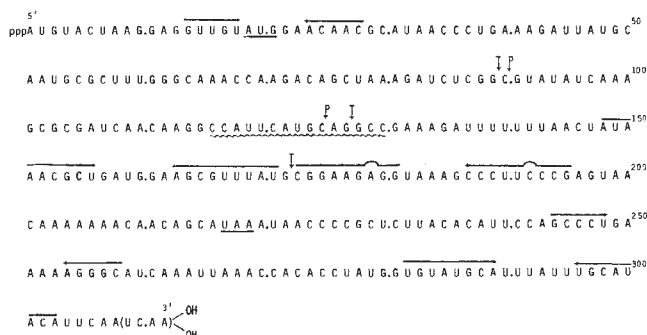
The synthesis of RNA catalysed by RNA polymerase from *Escherichia coli* is terminated at specific sites on DNA templates through the action of a multimeric basic protein known as  $\rho$  (refs 1, 2). Three lines of evidence suggest that an interactions of  $\rho$  with the nascent RNA is important for this termination. First,  $\rho$  binds strongly to RNA<sup>3</sup>; second,  $\rho$  expresses an RNA-dependent ATPase activity which is essential for termination<sup>4,5</sup>; third, RNA polymerase does not terminate RNA synthesis at  $\rho$ -dependent sites when the nascent RNA is digested by ribonuclease during transcription<sup>2,6</sup>. From the fact that certain RNAs, particularly single-stranded, pyrimidine-rich polymers containing at least 10% cytidylate residues, are more effective than other RNAs at promoting  $\rho$ -ATPase, it has been proposed<sup>7</sup> that  $\rho$  recognises specific sites on a nascent RNA for its action. We present evidence here that  $\rho$  does bind at a specific region on a mRNA transcribed from bacteriophage  $\lambda$  DNA.

A precisely located site where the synthesis of an RNA molecule is terminated by  $\rho$  action is at a region, called  $t_{R1}$ , that is 92-96 base pairs to the right of the ochre translation termination triplet for the *cro*-gene sequence on  $\lambda$  DNA<sup>8</sup>. Transcripts initiated at  $P_R$  on  $\lambda$  and terminated in this region are 308-312-nucleotide-long RNAs and sediment at 8-9S (ref. 9). We call these, collectively,  $\lambda$  *cro* mRNA, and its sequence, as deduced from published information on the sequences of the  $\lambda$  DNA in the area of the *cro* gene and the 5' and 3' ends of the RNA, is given in Fig. 1.

To study the binding of  $\rho$  to a specific site on  $\lambda$  *cro* mRNA, the RNA is prepared by transcription *in vitro* of a  $\lambda$  DNA fragment containing the sequences from the restriction nuclease *Hind*III site at position 77.6 (in gene  $C_1$ ) to the *Eco*RI site at position 81. This fragment actually codes for the synthesis of two RNAs *in vitro*,  $\lambda$  *cro* mRNA and  $\lambda$  *oop* RNA. From studies using a nitrocellulose filter binding assay, we have found that  $\rho$  forms a complex with  $\lambda$  *cro* mRNA, but not with  $\lambda$  *oop* RNA. We estimate that the association constant for the  $\rho$ - $\lambda$  *cro* mRNA interaction is of the order of  $10^8$  M<sup>-1</sup>. This binding is not high enough to isolate a fragment of RNA protected by  $\rho$  against

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**Fig. 1** The nucleotide sequence of  $\lambda$  *cro* mRNA. This sequence is based on published information<sup>8,11-13</sup>. Dots are placed after every 10 residues. The parenthesis at the 3' end indicates that termination occurs at any one of those bases. The AUG initiation codon and UAA termination codon for *cro* protein synthesis at residues 19-21 and 217-219, respectively, are underlined. The overlining with arrows designates some inverted sequence repetitions that might be involved in *cro* mRNA secondary structure. The symbols  $\uparrow$  and  $\uparrow$  indicate preferred sites of cleavage with  $T_1$  and pancreatic ribonucleases, respectively. The wavy underlining indicates the region where bond cleavage is inhibited by  $\rho$ .

ribonuclease digestion; however, it is high enough for  $\rho$  partially to protect sites on the RNA against cleavage by ribonucleases. We use this effect to demonstrate that  $\rho$  binds to a specific site on  $\lambda$  *cro* mRNA.

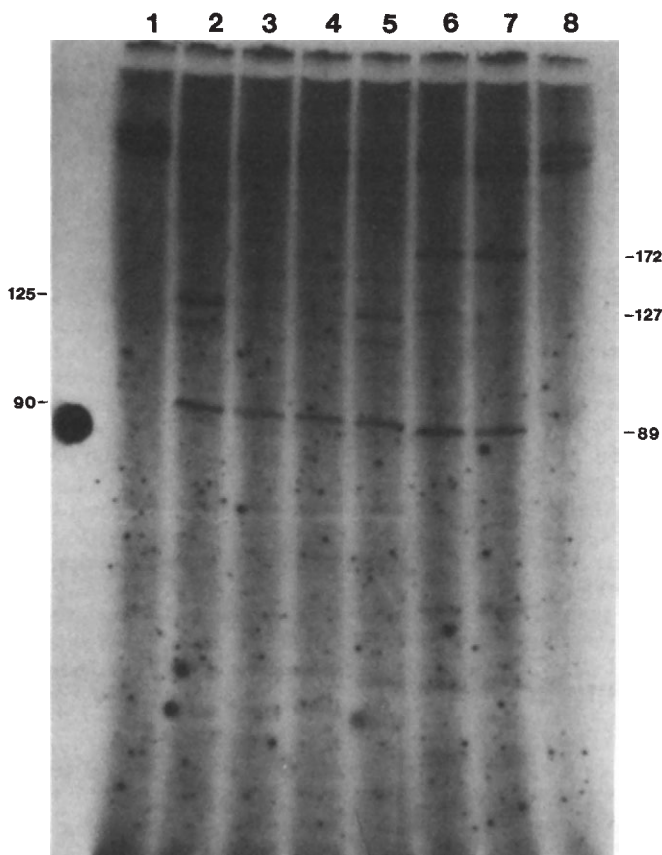
To simplify the task of locating cleavage sites on  $\lambda$  *cro* mRNA, we use RNA labelled at the 5' end and analyse the size distribution of its partial cleavage products by polyacrylamide gel electrophoresis. With this technique, the size of a labelled RNA fragment can be directly related to the point of cleavage on the primary structure of the *cro* RNA.  $\lambda$  *cro* mRNA labelled at the 5' end was prepared by transcription of the  $\lambda$  *cro*-gene DNA fragment *in vitro* (with  $\rho$  present) using [ $\gamma$ - $^{32}$ P]ATP. As  $\lambda$  *oop* RNA is initiated with GTP, the only labelled product is  $\lambda$  *cro* mRNA, which because of the heterogeneity in its termination, produces an unresolved group of bands on the gel rather than a single sharp band (Fig. 2, lanes 1 and 8). A partial digest of this RNA with pancreatic ribonuclease produces several distinct new bands, the two most prominent of which are at positions corresponding to RNA chain lengths of 90 and 125 nucleotides (lane 2). When the cleavage is carried out in the presence of a 10-fold molar excess of  $\rho$  over the RNA, many of the bands remain unchanged (lane 3), but the intensities of the bands from position 115 to position 129 are lower. Similarly, partial digestion of free  $\lambda$  *cro* mRNA with  $T_1$  ribonuclease produces three very distinct bands (lane 6), including one at position 127 that is totally absent when the digestion is carried out in the presence of  $\rho$  (lane 7). The inclusion of ATP in the reaction mixture has no effect on the pattern of bands formed nor on the sites protected by the presence of  $\rho$ . (Lanes 4 and 5 show the results with pancreatic ribonuclease digestion with and without  $\rho$  present, respectively; the results with  $T_1$  ribonuclease are not shown.)

The results show that there is a wide range of susceptibility of the bonds in  $\lambda$  *cro* mRNA to attack with ribonucleases. The most abundant products found after a limited partial digestion are the result of breakage at the most susceptible bonds. The very distinct pattern of products found is a clear indication that  $\lambda$  *cro* mRNA has a highly ordered secondary and perhaps tertiary structure. Presumably the most susceptible bonds are at exposed portions of the RNA. The presence of  $\rho$  clearly inhibits ribonuclease action at some susceptible points but not others. As  $T_1$  ribonuclease, pancreatic ribonuclease and  $\rho$  are all more active on single-stranded RNAs than on double-helical RNAs, we expect  $\rho$  to bind to an exposed portion of the RNA that would also be a preferred site for ribonuclease action. Thus, the very specific inhibition caused by  $\rho$  is probably the result of a direct blockage of a cleavage site by the bound  $\rho$  molecule, rather than the result of a conformational change at the cleavage site induced by the binding of  $\rho$  to some other sequence of the RNA.

Because there are so few sites that are highly susceptible to cleavage, this experiment gives only limited information on the

extent of the  $\lambda$  *cro* mRNA that could be in contact with  $\rho$ . The results suggest that there is some degree of contact with the region of residues 115-128, but that contact does not extend to position 88 on one side nor position 172 on the other side. With poly(C),  $\rho$  can bind up to 60 nucleotides in a single stretch<sup>10</sup>. However, as the binding of  $\rho$  to  $\lambda$  *cro* mRNA is several orders of magnitude weaker than its binding to poly(C), the extent of contact could involve many fewer nucleotides.

Based on an examination of the primary structure of  $\lambda$  *cro* mRNA, we predicted that a likely site for  $\rho$  binding would be the pyrimidine-rich segment of RNA on the part of the untranslated



**Fig. 2** Effect of  $\rho$  on the cleavage of  $\lambda$  *cro* mRNA with pancreatic and  $T_1$  ribonucleases. [ $\gamma$ - $^{32}$ P]-labelled  $\lambda$  *cro* mRNA was synthesised *in vitro* in a reaction mixture containing 5.6 pmol *E. coli* MRE 600 RNA polymerase holoenzyme, 5.6 pmol  $\rho$  protein, 1 pmol  $\lambda$  *cro* DNA fragment (77.6-81.0 map units of  $\lambda$  DNA), 0.1 mM each [ $\gamma$ - $^{32}$ P]ATP (10-20 Ci mmol<sup>-1</sup>), GTP and UTP, 0.05 mM CTP, 40 mM Tris-HCl, pH 8, 10 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1 mM dithiothreitol and 0.1 mM EDTA in a total volume of 0.1 ml. After incubation for 20 min at 37 °C, the RNA was purified by extraction successively with phenol and with a chloroform/isoamyl alcohol mixture (24:1) and precipitated with 2 volumes of 95% ethanol. After centrifugation for 30 min at 20,000g, the pelleted precipitate was washed in 0.5 ml 95% ethanol and the washed precipitate dissolved in a solution containing 10 mM Tris-HCl, pH 7.4, 10 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1 mM dithiothreitol, 0.1 mM EDTA, bovine serum albumin (50  $\mu$ g ml<sup>-1</sup>) and *E. coli* tRNA (1 mg ml<sup>-1</sup>). The samples analysed contained, in 10  $\mu$ l total volume, 0.1 pmol of [ $\gamma$ - $^{32}$ P] $\lambda$  *cro* mRNA (~10,000 c.p.m.). Samples with  $\rho$  contained 0.4  $\mu$ g and were preincubated for 1 min at 37 °C; ATP, when present, was 1 mM. Ribonuclease treatments at 37 °C were initiated by adding 1  $\mu$ l of either  $T_1$  ribonuclease (Sankyo-Calbiochem, 80 units ml<sup>-1</sup>) or pancreatic ribonuclease (Worthington, 5  $\times$  10<sup>-3</sup> units ml<sup>-1</sup>) and were terminated after 15 min by the addition of 1  $\mu$ l 1% diethyl oxydiformate. After adding 10  $\mu$ l of a solution containing 40% sucrose, 0.1% xylene cyanol and 0.1% bromophenol blue, the samples were applied to a 10% acrylamide gel (14 cm  $\times$  25 cm  $\times$  0.15 cm) containing 7 M urea in 90 mM Tris, 90 mM boric acid, 4 mM EDTA and 0.1% SDS. After electrophoresis for 5 h at 350 V, the lowest section of the gel containing residual unincorporated [ $\gamma$ - $^{32}$ P]ATP was cut away and the rest of the gel was washed in H<sub>2</sub>O for 0.5 h (to remove urea), dried and exposed to Kodak NS-2T X-ray film in the presence of a Cronex Lightning-plus intensifying screen at -70 °C. Lanes 1 and 8, untreated RNA; lanes 2-5, RNA treated with pancreatic ribonuclease.  $\rho$  was present in samples shown in lanes 3 and 4 and ATP was present in samples shown in lanes 4 and 5. Lanes 6 and 7, RNA treated with  $T_1$  ribonuclease in the absence and presence of  $\rho$ , respectively. The dark spot at the left marks the position of the xylene cyanol dye.

tail of the RNA that starts after the ochre chain termination triplet at position 220 and runs to the start of the proposed hairpin loop structure (*nutR* [see ref. 8]) at position 244. By itself, this region would probably have very little secondary structure. Although there is evidence that bonds from residues 220 and 244 are cut with pancreatic ribonuclease, the sites are not as sensitive as those at positions 90 and 125, and the single G residue at position 228 is resistant to  $T_1$  ribonuclease action. Thus, it is likely that this stretch of RNA is protected by folding of the RNA. As  $\rho$  has no obvious effect on the activity of pancreatic ribonuclease in that region, it may be that the folding of the RNA prevents it from being an effective  $\rho$  binding site as well.

The results presented here indicate that  $\rho$  does bind to a specific site on  $\lambda$  *cro* mRNA and that is within the sequence coding for the *cro* protein 180 residues from the 3' end rather than on the untranslated tail. In the current models<sup>2,7,10</sup>, it is proposed that  $\rho$  protein functions in termination primarily to effect the release of RNA molecules from a transcription complex in which the RNA polymerase has paused at a termination site on the DNA template. As the sites for pausing are apparently determined by the interaction between the RNA polymerase, DNA and the nascent RNA<sup>8</sup>, the initial site of attachment of the release factor need not be at a sequence adjacent to the 3' end. Thus, the binding site detected in this study could be relevant to  $\rho$  function in termination. However, because the structure of RNA is very sensitive to the environment, which is not the same for a nascent RNA as it is for an isolated RNA, we cannot be certain that  $\rho$  binds to the same region on the nascent RNA. Hence, although we have identified for the first time a specific site for the interaction between  $\rho$  and a mRNA, further experiments will be needed to show whether this site is recognised by  $\rho$  during the process of transcription termination.

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## Potential of *Escherichia coli* isolated from nature to propagate cloning vectors

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Concern has been shown in the scientific community with regard to the potential risk that the transmission of foreign genes from *Escherichia coli* K12-cloning vector systems to other microorganisms might represent<sup>1</sup>. Because of this we have attempted to evaluate the frequency in nature of *E. coli* strains that can serve as recipients and/or hosts for genetic information

harboured by various *E. coli* cloning vectors used in recombinant DNA research. We assumed that genetic transfer from *E. coli* K12 to other *E. coli* strains was more likely than to bacteria of other species or genera, and consequently examined natural *E. coli* isolates for traits that would reflect their ability to propagate and/or maintain cloning vectors. In particular, we were interested in assessing the role that transduction might have in the dissemination of cloned genetic information. We also wanted to determine whether naturally occurring strains could acquire and stably maintain phage  $\lambda$  derived cloning vectors that generally contain *amber* mutations in essential genes<sup>2-4</sup>. By examining natural bacterial populations we attempted to evaluate the fitness of auxotrophic mutations that are generally introduced into *E. coli* K12 hosts used in recombinant DNA experiments. Therefore, we determined among natural isolates of *E. coli* frequency of strains exhibiting: (1) sensitivity to phages P1 and  $\lambda$ ; (2) *amber* suppressor mutations; and (3) auxotrophic mutations. The results presented here allow an estimation of the feasibility and limitations of the successful spread among *E. coli* strains in nature of recombinant DNA molecules contained in particular *E. coli* K12 hosts.

In terms of propagation of cloning vectors, we considered it important to evaluate the possibility that they might be transferred to and among natural *E. coli* strains through generalised transduction by a phage like P1. Although P1 is known to have a wide host range<sup>5,6</sup>, the prevalence of P1-mediated transduction in nature, and the incidence of P1 sensitivity among natural isolates of *E. coli* are not known. We studied this last parameter for an indication of the importance which P1 transduction might have on the dissemination of plasmid cloning vectors among naturally occurring bacteria. Sensitivity to P1 of the *E. coli* isolates studied was determined using the phage derivative P1CMclr100 (ref. 7) which confers resistance to chloramphenicol (Cm) to lysogenised cells. A substantial majority of the *E. coli* strains studied (about 70%) were found to be sensitive to P1 (Table 1, c). Generally these strains were lysogenised by P1CMclr100 at frequencies ranging from  $10^{-2}$  to  $10^{-4}$ .

In the case of  $\lambda$  cloning vectors an inherent safety feature seems to derive from the low incidence of sensitivity to  $\lambda$  found among natural isolates of *E. coli* using a plaque formation assay<sup>4</sup>. However, these studies may have underestimated the sensitivity to  $\lambda$  of those strains, as plaque formation requires successful propagation of the phage and is therefore affected by factors such as reduced adsorption, restriction and/or the presence of a homoimmune phage<sup>8</sup>, all of which will reduce the plating efficiency of  $\lambda$ . Considering these limitations to using lysis as a test for  $\lambda$ -sensitivity, we decided to examine our *E. coli* isolates for sensitivity to phage  $\lambda$  using the thermoinducible derivative  $\lambda$ cam105 (ref. 9) which contains in its genome the determinant for Cm resistance, Tn9.

By these means, penetration of  $\lambda$  into  $\lambda$ -sensitive *E. coli* cells can be detected by the acquisition of a Cm-resistant phenotype that might result from lysogenisation by  $\lambda$ cam105 and/or by transposition of Tn9 to the chromosome, or a resident plasmid, or phage of the strain being studied. About 2% of the *E. coli* strains tested yielded Cm-resistant clones when infected with  $\lambda$ cam105 (Table 1, d). These clones were analysed further and it was found that in eight out of nine instances, the Cm-resistant derivatives could grow at 30°C but not at 42°C. This indicated that they were lysogens of  $\lambda$ cam105 since the parental strains showed unimpaired growth at 42°C. In addition, lysates prepared from the Cm-resistant derivatives which were thermosensitive could only transduce to Cm resistance *E. coli* K12 strains that were not  $\lambda$ -lysogens. The frequency of lysogenisation by  $\lambda$ cam105 was estimated to be between  $10^{-3}$  and  $10^{-4}$ . The strain that gave rise to Cm-resistant clones which were not thermoinducible might represent a case in which  $\lambda$  was lost after infection, and the Cm-resistant phenotype resulted from a Tn9 transposition event. We noted that the  $\lambda$  derivative  $\lambda$ vir (ref. 10) did not form plaques on any of the strains shown to be infectable by  $\lambda$ cam105. This indicates that a plaque assay is generally not

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Table 1 Characteristics of natural isolates of *Escherichia coli*

| Origin <sup>a</sup> | No. of isolates | Fraction of isolates                                                           |                                                        |                                                  |        |
|---------------------|-----------------|--------------------------------------------------------------------------------|--------------------------------------------------------|--------------------------------------------------|--------|
|                     |                 | <i>P1</i> <sup>c</sup><br>sensitive to phages<br>and<br>$\lambda$ <sup>d</sup> | with <i>amber</i> suppressor<br>mutations <sup>f</sup> | containing auxotrophic<br>mutations <sup>i</sup> |        |
| Infections          | 325             | 212/310                                                                        | 2/237                                                  | 2/254 <sup>g</sup>                               | 37/325 |
| Stools              | 190             | 135/180                                                                        | 7/143                                                  | 0/154                                            | 11/188 |
| Polluted water      | 35              | 20/23                                                                          | 0/34                                                   | 0/35                                             | 3/35   |
| Totals              | 550             | 367/524                                                                        | 9/414 <sup>e</sup>                                     | 2/443 <sup>h</sup>                               | 51/548 |
| (%)                 |                 | (70.0)                                                                         | (2.1)                                                  | (0.4)                                            | (9.3)  |

a, *E. coli* strains from infections were obtained from CDC stocks (Atlanta, Georgia) and additional strains were isolated from specimens collected at the UAB hospitals. All strains from infections were of human origin. Strains from faecal samples from both healthy and diseased human individuals were also obtained from UAB hospitals and CDC, respectively. *E. coli* strains were isolated from polluted water samples collected from wells and water systems around the Birmingham area. The strains from CDC (328 isolates) had been originally collected between 1972 and 1976. In most cases, the O, H and K antigenic types had been determined. Strains from UAB (187 isolates) and from polluted waters were collected during 1977. No serotyping was done with these strains.

b, The total number of isolates includes strains that were resistant to one or more of the following drugs: Ap, Tc, Km and Cm (Sigma). Some of these strains, depending on the resistance(s) they displayed, were not tested for *amber* suppressors and/or sensitivity to phages P1 and  $\lambda$  due to experimental design.

c, Lysates of P1CMclr100 were prepared by thermoinduction of the *E. coli* K12 P1CMclr100 lysogen  $\chi$ 1932 (F<sup>-</sup>tsx  $\lambda$  *nalA* *rpsL*). *E. coli* strains to be tested were grown at 37 °C to a density of  $1 \times 10^8$  cells ml<sup>-1</sup> in L broth<sup>13</sup> supplemented with  $2.5 \times 10^{-3}$  M CaCl<sub>2</sub>. Cultures were infected with P1CMclr100 at room temperature using the phage at a multiplicity of infection of 1. After allowing 20 min of preadsorption at 30 °C, samples of the infected cultures were plated on Penassay agar (PA, Difco) supplemented with 12.5  $\mu$ g ml<sup>-1</sup> Cm. After 24–36 h of incubation at 30 °C, plates were examined for the presence of Cm-resistant lysogens.

d, Lysates of  $\lambda$ c1857cam105 were prepared by thermoinduction of its *E. coli* K12 host MGBO (F<sup>-</sup>*thi rha trkA*<sub>hkd</sub>) (provided by L. A. MacHattie).  $\lambda$ cam105 was then used to detect  $\lambda$ -sensitive strains using the same procedure described for P1-sensitivity screening, except that the cells were grown in L broth without glucose and supplemented with  $1 \times 10^{-2}$  M MgSO<sub>4</sub>. Also, selective plates contained 20  $\mu$ g ml<sup>-1</sup> Cm.

e, Strains lost on storage at 4 °C account for the lower number of isolates tested for  $\lambda$ -sensitivity in relation to the number examined for P1-sensitivity.

f, See text for experimental rationale. *E. coli*  $\chi$ 1776 (F<sup>-</sup> *tonA53 dapD8 minA1 supE42*  $\Delta$ 40[*gal-uvrB*]  $\lambda$  *minB2 rfb-2 nalA25 oms-2 thyA142 metC65 oms-1*  $\Delta$ 29[*bioH-asd*] *cycB2 cycA1 hsdR2*)<sup>14</sup> containing the plasmid pLM2 [Ap(am) Tc(am) Km]<sup>15</sup> was used as donor in matings with the natural *E. coli* isolates tested.  $\chi$ 1776 (pLM2) and the recipient strain under examination were grown to a density of  $1 \times 10^8$  cells per ml in L broth supplemented with 100  $\mu$ g diaminopimelic acid (DAP) and 40  $\mu$ g thymidine (Thd) per ml. Samples (0.1 ml) of donor and recipient were mixed on PA plates containing DAP and Thd and incubated overnight at 37 °C. Generally, the recipient strain was heat shocked before mating to minimize restriction of the incoming plasmid<sup>15</sup>. After incubation, the mating mixture was resuspended in BSG<sup>16</sup> and samples were plated onto PA containing 100  $\mu$ g ml<sup>-1</sup> Km to select pLM2 transconjugants. Selection against the donor was achieved by omitting DAP and Thd in the PA Km plates. A minimum of 5 pLM2 transconjugants of each natural *E. coli* strain tested were examined for their ability to grow in the presence of Ap (25  $\mu$ g ml<sup>-1</sup>) and/or Tc (25  $\mu$ g ml<sup>-1</sup>) to determine if the *E. coli* strain being studied contained an *amber* suppressor function.

g, The two strains showing *amber* suppressors were of human origin and had been originally recovered from infectious episodes of the urinary tract and blood. The strains were stocked at CDC with the numbers 3764–73 (urine isolate) and 3885–74 (blood isolate). Their serotypes were O undetermined: H4 and O16: H6, respectively. Both were prototrophs, P1-sensitive and  $\lambda$ -resistant. Both strains constituted homogeneous *amber* suppressor populations.

h, No. of strains into which pLM2 was successfully transferred to test for *amber* suppressors. The actual number of isolates that could have been tested for suppressors was 486 out of the 550 originally collected.

i, The auxotrophic character of the strains examined was determined by their inability to grow on minimal agar<sup>16</sup> plates with 1% glucose as carbon source.

sensitive enough to determine whether a particular *E. coli* strain can be infected by  $\lambda$  or not.

It was mentioned above that phage  $\lambda$ -derived cloning vectors generally possess *amber* mutations in essential genes<sup>2–4</sup>. It has also been proposed (but not yet accomplished) to construct non-conjugative plasmid cloning vectors with the same type of mutations. The purpose of constructing vectors with *amber* mutations is to confine their full expression to an *E. coli* K12 host with an *amber* suppressor. This approach to the development of safer vectors for cloning was obviously based on the assumption that the majority of bacterial strains found in natural environments would not contain *amber* suppressors and thus would not propagate cloning vectors should they accidentally acquire them. However, no data are available on the incidence of natural bacterial strains possessing *amber* suppressor mutations. We sought to detect such mutations among the *E. coli* isolates collected using the plasmid pLM2 (ref. 11). This plasmid is a derivative of RP1, a broad host range plasmid that specifies resistance to ampicillin (Ap), tetracycline (Tc) and kanamycin (Km). pLM2 differs from RP1 in that the Ap and Tc markers have *amber* mutations. Consequently, these antibiotic resistances are expressed only in a host strain that contains an *amber* suppressor. Therefore, pLM2 was used to detect *amber* suppressors by transferring it to the test strains and verifying whether resistance to Ap and Tc was expressed. We could transfer pLM2 to 91% of the strains that were suitable to be tested for *amber* suppressors (Table 1, f, h). Only two *E. coli* strains (representing about 0.4% of the total examined) were found containing *amber* suppressors. Both suppressor strains were detected among a group of isolates obtained from the Center for Disease Control (CDC), Atlanta, Georgia (see Table 1 g, for additional information).

Generally, *E. coli* K12 derivatives used as hosts in recombinant DNA experiments have specific auxotrophic mutations that result in a decrease in viability of the strains when they are

placed in laboratory conditions that mimic a natural environment. Examples of mutations that severely hinder survival of *E. coli* K12 derivatives are those that lead to the requirement of cell wall components like diaminopimelic acid and/or precursors of nucleic acids like thymidine. An independent way of assessing the effect of such mutations on the survival of a particular natural strain is to determine whether the corresponding phenotypes are present among natural bacterial isolates. Therefore, we tested our *E. coli* isolates for the presence of auxotrophic mutations and whenever possible the nature of the metabolic requirement was determined. We found that approximately 9% of the natural *E. coli* strains studied were auxotrophs (Table 1), a result that is in agreement with available information<sup>12</sup>. When further analysed for the nature of their nutritional requirement, 77% of the auxotrophic strains were found to require niacin. The rest required either a different vitamin (thiamine, pantothenate) or certain amino acids, namely cysteine or methionine. No purine or pyrimidine requirers were found. We noted that a group of *E. coli* isolates from urinary tract infections showed the highest incidence (25%) of auxotrophic strains.

The results of this investigation suggest that P1-mediated transduction might be an important mechanism for dissemination of genetic information in nature in view of the high incidence of P1-sensitive strains found among the *E. coli* isolates that were examined. This observation is consistent with the broad host range of P1 (refs 5, 6). However, additional experiments are necessary to evaluate further the possible role of P1 in the transmission of genetic material between natural bacterial populations. It would be desirable to determine titres of P1-like phage in nature, either as a free phage or as a prophage in lysogenic cells; in addition, stability and infectivity data of P1-like phage particles in natural conditions would be required. This information would be important in view of results that show that P1 is able to transduce plasmids similar to those

used as cloning vectors, although at a lower frequency than for chromosomal genes (R. Goldschmidt and M. Inoue, unpublished).

In terms of  $\lambda$  cloning vectors, our observations indicate that these cloning vehicles have inherent safety features that derive from the relatively low incidence of  $\lambda$ -sensitive strains among naturally occurring strains of *E. coli*. From the results that indicate the presence at low frequency of *amber* suppressor mutations among the *E. coli* isolates examined, it is apparent that cloning vectors with *amber* mutations in essential genes contained in bacterial hosts with suppressors, offer a safety advantage over vectors lacking *amber* mutations. We estimate that the probability of successful expression of an *amber*-containing cloning vehicle would be reduced at least 100-fold when compared to that of a vector without such genetic lesions in its genome.

The fact that we were able to detect auxotrophs among the bacterial strains tested suggests that certain vitamin and amino acid requirements do not necessarily represent a selective disadvantage to the bacteria that possess them. On the other hand, the diversity of auxotrophic mutations was rather limited and it seems unlikely that all possible types of auxotrophs will be found among natural bacterial isolates. In particular and as expected, we did not find any of the phenotypes associated with mutations that result in a severe impairment of the ability of *E. coli* K12 derivatives to survive in natural environments.

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## Anisotropy of Young's modulus of bone

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Bonfield and Grynias<sup>1</sup> have compared their experimental data for Young's modulus of elasticity versus the angle of orientation of the specimen to the long axis of bone with a theoretical curve predicted from a calculation for fibre reinforced materials proposed by Currey<sup>2</sup>. As a result of the poor agreement between the two curves they conclude "... an alternative model is required to account for the dependence of Young's modulus on orientation" (ref. 1). Such an alternative has been under development in my laboratory for the past 8 years (refs 3-7). It is a two-level hierarchical fibre-reinforced composite model which appears to provide a much more suitable description of the behaviour of bone as a fibre-reinforced composite material.

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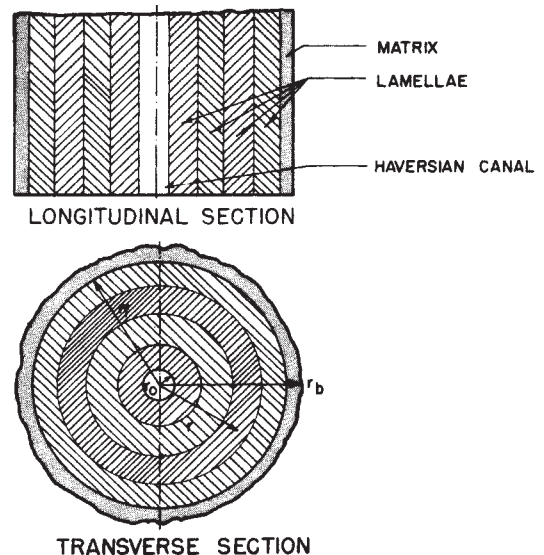


Fig. 1 A single osteon modelled as a hollow fibre for the Hashin-Rosen<sup>9</sup> calculation.  $r_0$  is the radius of the Haversian canal (hollow),  $r_1$  is the radius of the single osteon (fibre) and  $r_b$  is the radius of the osteon plus cement line (fibre and matrix).

At the first level of the model, a single osteon is treated as a coherent entity, modelled as a hollow, right circular cylinder comprised of concentric lamellae surrounding the Haversian canal (Fig. 1). On the second level the osteons or interstitial lamellae are assumed to be packed in a pseudo-hexagonal arrangement (Fig. 2), as suggested for compact cortical bone by Yoon and Katz<sup>8</sup> based on microscopic observations and ultrasonic wave propagation measurements.

The Hashin-Rosen calculation<sup>9</sup> for hexagonally and randomly (nearly hexagonally) arrayed hollow fibre-reinforced composites can now be applied directly. In this model only five independent effective elastic constants are required to characterise completely the anisotropic elastic properties of the material, either exactly as in the case of hexagonal arrays or approximately for random (nearly hexagonal) arrays. Variational techniques provide a set of bounds for the five effective

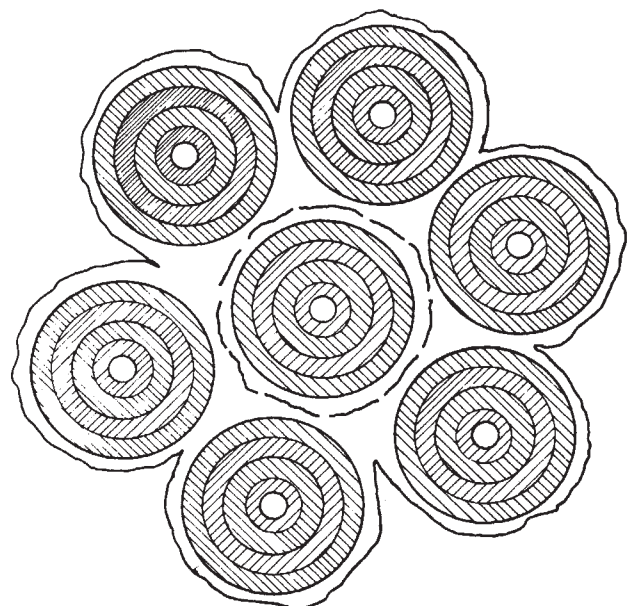
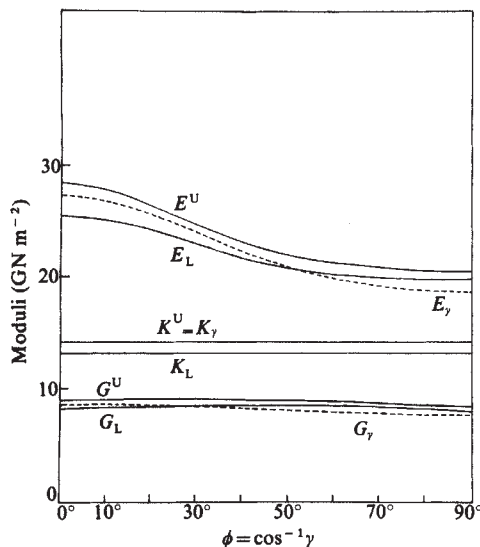


Fig. 2 Transverse cross-section of seven hollow fibres in a near-hexagonal array modelling a specimen of cortical Haversian bone. (The number of lamellae depicted for each osteon is for artistic purposes only and does not affect the calculation.)



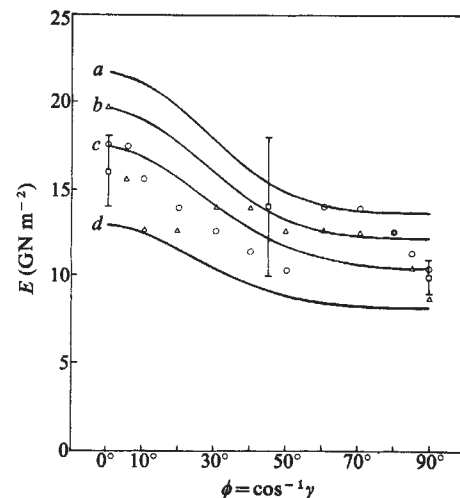
elastic constants; the upper bounds are obtained by using displacement criteria, the lower bounds by using the stress-vector criteria.

Two kinds of input are required for this calculation. First, geometric factors are needed that include the ration of the transverse area of the haversian canal to that of the osteon, given by  $r_o^2/r_i^2$  (Fig. 1) and denoted by  $V_o$ , and the ratio of the transverse area of the osteon to osteon plus matrix, given by  $r_i^2/r_b^2$  (Fig. 1) and denoted by  $V_i$ . Second, mechanical properties are needed that include the Young's modulus and Poisson's ratio for both the fibre represented here by the single osteon ( $E_{OST}$  and  $\nu_{OST}$  respectively) and the matrix represented here by the ground substance ( $E_{GS}$  and  $\nu_{GS}$  respectively). Reported values of  $E_{OST}$  under various ultrastructural configurations<sup>4</sup> agree well with values for single osteons measured by Frasca<sup>10</sup>; values of  $\nu_{OST}$  have also been derived from his data. Values for the ground substance have been inferred from the angular dependent properties of bone obtained from ultrasonic experiments<sup>8</sup>.



**Fig. 3** The angular dependent variation of the elastic moduli for human cortical bone with respect to the orientation of the long axis of the bone ( $\phi$ ) calculated from the ultrasonic data of Yoon and Katz<sup>8</sup> (Young's modulus,  $E_r$ ; bulk modulus,  $K_r$ ; and shear modulus,  $G_r$ , are denoted by ---) compared with the theoretical curves representing the upper and lower bounds on the angular dependent behaviour of the elastic moduli using the Hashin-Rosen calculation<sup>9</sup>. (Young's moduli,  $E^U$ ,  $E_L$ ; bulk moduli,  $K^U$ ,  $K_L$ ; and shear moduli,  $G^U$ ,  $G_L$  respectively are denoted by —.) Input parameters are:  $E_{OST} = 40.0 \text{ GNm}^{-2}$ ;  $E_{GS} = 10.0 \text{ GNm}^{-2}$ ;  $\nu_{OST} = 0.30$ ;  $\nu_{GS} = 0.25$ ;  $V_o = 0.10$  and  $V_T = 0.70$ .

These ultrasonic measurements have yielded five independent elastic stiffness constants which also can be used to calculate the technical moduli as a function of the angular orientation with respect to the stress axis. The angular dependence of the Young's  $E$ , bulk,  $K$ , and shear,  $G$ , moduli calculated from the ultrasonic data are compared on Fig. 3, with a set of upper and lower bound curves generated by using the best pair of five effective elastic constants obtained from a large number of sets computed using the Hashin-Rosen formalism<sup>9</sup>. The values of these ultrasonic moduli for dry bone are all greater than those obtained by mechanical testing or low frequency sound measurements on wet bone (Fig. 4). This is due partly to the fact that dry bone is stiffer and more rigid than the same sample of bone when wet, and partly to the strain rate dependent behaviour of bone which results in increased stiffness with increase in strain rate<sup>11</sup>. Several groups of such curves have been analysed in detail to obtain representative sets of values for both the Young's modulus and Poisson's ratio of the matrix based on



**Fig. 4** The variation of Young's modulus for bovine femoral specimens with respect to the orientation of specimen axes cut at the angle  $\phi$  to the long axis of the bone for wet (○) and dry (Δ) conditions from the work of Bonfield and Grynpas<sup>1</sup> compared with the angular dependent behaviour of Young's modulus (—) computed using the Hashin-Rosen calculation<sup>9</sup>. Each curve represents a different lamellar configuration of collagen fibres and hydroxyapatite crystallites within a single osteon.  $a$ , 65% longitudinal fibres;  $b$ , 57% longitudinal fibres;  $c$ , 50% longitudinal fibres;  $d$ , 37% longitudinal fibres. Other input parameters are:  $E_{GS} = 5.5 \text{ GNm}^{-2}$ ;  $V_o = 0.20$ ; and  $V_T = 0.80$ .

scaling the ultrasonic data to the mechanical and low frequency sound measurements.

Various combinations of these parameters have been used in calculations, based on the Hashin-Rosen formalism, which then generate sets of the five effective elastic constants. These constants are then used to calculate the angular dependence of such technical moduli as the Young's and shear moduli as a function of angular orientation with respect to the stress axis. These calculations have been used as the basis for determining which sets of parameters were used in generating curves  $a-d$  (Fig. 4) in order to model the data of Bonfield and Grynpas. Additional confirmation of this type of angular dependent behaviour for the elastic properties of bovine bone is shown in Fig. 4. The values measured for bovine bone specimens cut at 0°, 45° and 90° to the bone axis (denoted by the open squares with error bars) are from ref. 12.

Although this model simulates the behaviour of bone quite closely it cannot provide an exact description. It handles only one size of osteon in a given calculation. However, it can be adjusted to yield an average behaviour or to predict the upper and lower bounds on the elastic behaviour by the choice of lamellar micro- and ultrastructure (number of lamella, size of the haversian canal, fibre orientations and so on) as is shown in part by Fig. 4.

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# MATTERS ARISING

## Rotating lunar globules

SEVERAL theories have been proposed for the origin of the shapes of lunar dumbbell and ellipsoidal glass globules. The rotational theory<sup>1</sup> assumes that they solidify while rotating in free fall above the lunar surface, the rotation giving the elongation in form. A second theory imagines solidification of a jet of liquid again above the lunar surface and at some time during the break-up of the jet<sup>2-4</sup>. More recently Chernyak and Nussinov<sup>5</sup> have proposed that the shapes are caused by the sudden freezing of a vibrating droplet. They also cite arguments against a rotational origin. However, there is now much evidence favouring the rotational hypothesis.

Chernyak and Nussinov propose that "the drops vibrate" and "because of the exponential dependence of viscosity on temperature the particle solidifies and memorises its shape". Such a proposal is completely untenable as a glass drop cannot pass from an essentially liquid to solid state in a time that is short compared with its vibrational period. The period of vibration of a droplet of diameter 100  $\mu\text{m}$  is about  $10^{-4}$  s. Even assuming it radiates as a perfect black body the drop in temperature of such a globule during its vibrational period is between 0.1 and 0.01 K. Although the viscosity of glasses varies very rapidly with temperature (three orders of magnitude for a temperature change of 100 K) it is impossible that there can be any appreciable change in viscosity during a single period of oscillation, let alone during the small fraction of the period required by the vibrational hypothesis. Similar numerical analyses for larger or small droplets yield essentially the same conclusion because the ratio of the vibrational period to the time taken to cool through a given temperature interval varies only slowly with particle size<sup>1</sup> (in fact, as the square root of its linear dimensions).

Arguments by Chernyak and Nussinov<sup>5</sup> against the rotation hypothesis depend on the instability of dumbbell shapes and the effect of Coriolis forces which would bring about S or crescent shaped particles. Although several curved smooth glassy particles have been found in lunar soil and it is reasonable to use a Coriolis force mechanism to explain their shape, if the initial temperature of a particle is sufficiently high, the cooling time is long enough for viscous forces to damp out all appreciable internal motion. While still liquid the globule will thus eventually rotate as a rigid body<sup>1</sup> and Coriolis forces are then not involved.

Chernyak and Nussinov's argument that dumbbell shapes are unstable is also

not substantial. Imagine a small liquid sphere in an inertial frame held together only by surface tension forces. Suppose a small external couple acting for a limited time causes the globule to be brought into rotation. After the couple has been removed the globule will attain an aspherical rotating form in which any internal motions will gradually be eliminated by viscous damping. The form of the globule is then stable yet not spherical. The process may be repeated so that more and more angular momentum will be slowly given to the globule. Eventually the system will disintegrate but it is unclear whether before this disintegration occurs stable forms may exist with re-entrant shaped cross-sections—dumbbells.

There are many other reasons for preferring the rotational hypothesis. (1) The photographed shapes of lunar dumbbells<sup>6</sup> are not the same as those formed by jet break-up<sup>7</sup>. The latter have low curvature at their extremities. (2) The curvature of the surfaces of lunar dumbbells has been shown to agree well with that predicted from rotational theory<sup>6</sup>. (3) The break-up of a jet is a transitory phenomenon: a rotating dumbbell is a stable form so that the latter would be expected to occur more frequently. (4) There is no evidence that regular cylindrical liquid jets can be formed by volcanic activity or hypervelocity impacts. Such a jet is required by the jet break-up hypothesis<sup>2,3</sup>. Some evidence does exist for jet formation following meteorite impact<sup>8</sup> but here 'jet' simply implies a group of particles moving with high speed in approximately the same direction. The confusion is semantic. (5) The angular momentum of dumbbells and prolate globules is well accounted for by local inhomogeneities in surface rock at the impact site. (6) Voids both in the dumbbells and the oblate spheroids tend to be found close to the rotational axis, whereas darker and more dense particles are found closer to the extremities<sup>9</sup>. Generally more migration has occurred in the case of the larger particles and voids; and this is precisely what would be expected from Stokes' law and the rotational hypothesis. (7) The densities of the dumbbell extremities are found to be higher than the central portions, a result predicted by the rotational hypothesis (T. Gold, personal communication).

The rotation hypothesis supplies a means of determining the maximum temperature of droplets during their formation. It also favours a lava fountain origin for many of the green and orange lunar droplets as no dumbbell-shaped globules have been reported among these samples<sup>10</sup>. It is also relevant to the question raised by Butler<sup>11</sup> concerning

whether these droplets were splashed from molten lava by impact.

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## Dihydroergocryptine binding and $\alpha$ -adrenoreceptors in smooth muscle

THE recent letter by Kunos *et al.*<sup>1</sup> in which they "dissociate phentolamine-suppressible <sup>3</sup>H-dihydroergocryptine (<sup>3</sup>H-DHEC) binding and  $\alpha$ -receptors" in rabbit myometrium disagrees with previous reports<sup>2,3</sup> and contains several puzzling features.

First, the authors assume that the concentrations of phenoxybenzamine (POB) inhibiting noradrenaline-stimulated contractility should coincide with concentrations of POB that compete for <sup>3</sup>H-DHEC binding sites. As the number of steps between receptor binding and contractility is unknown, one ought not necessarily expect such different experimental approaches to yield similar results<sup>4,5</sup>. Moreover, it is probably unreasonable to ascribe the effects of a partially irreversible  $\alpha$ -adrenergic blocker (POB) on contractility solely to blockade of  $\alpha$ -adrenergic receptors, because POB also inhibits response to serotonin, histamine and acetylcholine<sup>6</sup>. Second, the authors suggest that two classes of <sup>3</sup>H-DHEC binding site ( $K_d$  values 1.5 and 5.5 nM) exist in their preparations. The data used to determine those  $K_d$  values were apparently obtained from pooled determinations of different uterine preparations. In view of the likely heterogeneity between particulates of crude myometrial homogenates subjected to



five to seven centrifugations (Fig. 1 legend<sup>1</sup>), selective pooling of such results is an unreasonable approach for determining two classes of site with such similar  $K_d$  values.

Third, we find that the concentration curve of <sup>3</sup>H-DHEC binding for 'control' values plotted by Scatchard analysis of these data yields a single class of <sup>3</sup>H-DHEC site ( $K_d = 4.6$  nM,  $B_{max} = 85.9$  fmol per mg,  $r = 0.93$ ). Even if the authors were correct in defining two classes of site, we calculate that in experiments carried out at 2 nM, their "high affinity sites" contribute 55% and the "low affinity sites" 45%, and in studies at 8 nM, the high and low affinity sites contribute 35% and 65%, respectively. We have constructed theoretical binding curves for two-non-cooperative sites with values for  $K_d$  and  $B_{max}$  described by the authors and find no break such as shown by Kunos *et al.* Although positive cooperativity at the low affinity class of sites might generate such a break, we have never seen such positive cooperativity in over 50 <sup>3</sup>H-DHEC binding studies with rabbit myometrium preparations.

Moreover, as binding at "high" (>5 nM <sup>3</sup>H-DHEC) concentrations is the sum of high and low affinity binding sites, the reported inhibition of <sup>3</sup>H-DHEC binding by POB only at concentrations below 4 nM <sup>3</sup>H-DHEC cannot be readily explained. Finally, our previous paper was misquoted. Attention to the reported data would indicate that oestrogen increased <sup>3</sup>H-DHEC binding at all concentrations from 0.5 to 100 nM <sup>3</sup>H-DHEC<sup>3</sup>.

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THE report by Kunos *et al.*<sup>1,2</sup> on the characteristics of tritiated dihydroergocryptine (<sup>3</sup>H-DHE) binding to rabbit uterine  $\alpha$ -adrenergic receptors suggested that (1) DHE binds with differential affinity to two classes of  $\alpha$ -adrenergic binding sites ( $K_d$  values of 1.5 and 5.5 nM,

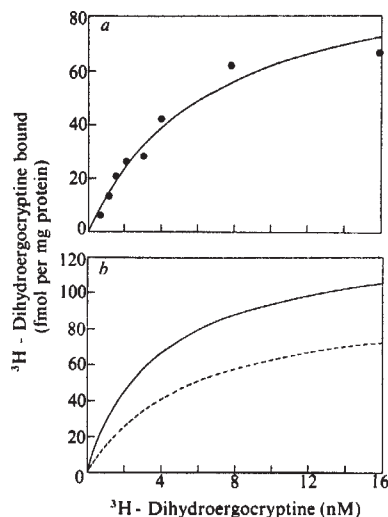


Fig. 1 *a*, Computer modelling of the control binding curve from Kunos *et al.* (Fig. 2, ref. 1). The solid circles represent graphical estimates of the binding data obtained by computerised digitisation of the published figure. The solid line is the best fit to a model for one class of binding sites according to the mass action law. The data could not be successfully fitted to a two-site model.  $K_d$  = dissociation constant of receptors for <sup>3</sup>H-DHE =  $6.2 \pm 1.4$  nM,  $R$  = receptor concentration =  $101 \pm 14$  fmol per mg. *b*, Computer simulation of a control (solid line) and a POB-treatment (dashed line) binding curve, based on parameter estimates provided by Kunos *et al.*<sup>1,2</sup>; two classes of binding site ( $K_d = 1.5$  nM and 5.5 nM,  $R = 37$  and 97 fmol per mg protein) for the control curve and one class of binding site ( $K_d = 5.5$  nM and 97 fmol per mg protein) for the POB-treatment curve (compare with Fig. 2, ref. 1).

respectively); (2) phenoxybenzamine (POB), at a concentration which completely blocks the contractile response to the  $\alpha$ -agonist noradrenaline, also selectively blocks the higher affinity class of  $\alpha$ -adrenergic binding sites (those presumably labelled at DHE concentrations  $\leq 3$  nM) while having no effect on the lower affinity sites (those presumably labelled at DHE concentrations  $> 3$  nM).

The first conclusion which is seemingly at variance with published reports from several laboratories<sup>3,4,5</sup>, is based on the observation of a break in the hand-drawn curve of a binding isotherm for <sup>3</sup>H-DHE (Fig. 2, ref. 1). We obtained graphical estimates of their binding data and subjected them to computer modelling, using well established methods<sup>6-9</sup>. Figure 1*a* (compare with Fig. 2, ref. 1) shows that their data were best fitted with a model for one class of DHE binding sites ( $K_d = 6.2 \pm 1.4$  nM,  $R = 101 \pm 14$  fmol per mg protein). Attempts to fit the experimental data with a model for two classes of DHE binding sites were unsuccessful in providing parameter estimates, indicating that the data did not support the hypothesis of more than one class of DHE binding site. Computer modelling of the first five points (DHE  $\leq 3$  nM) of their binding isotherm provided similar parameter estimates for one class of DHE binding site although much less accurately ( $K_d = 6 \pm 6$  nM,  $R =$

$90 \pm 60$  fmol per mg), again in contrast with the results of their subjective graphical analysis of these early points. In agreement with the findings of other investigators<sup>3-5</sup> and in conflict with their own interpretation, the data of Kunos *et al.* actually indicate the presence of a single homogeneous population of DHE binding sites in rabbit uterine membranes.

Despite the inability of their experimental data to support objectively the hypothesis of two classes of DHE binding site, the parameter estimates reported by Kunos *et al.*<sup>1,2</sup> for these two putative classes of DHE binding site could be used to provide computer simulations (Fig. 1*b*) of control and POB-treatment curves. Such simulations should reproduce the salient features of the data as interpreted by Kunos *et al.*—the presence of a break in the binding isotherms and a difference in the control and POB-treatment curves only at low DHE concentrations (DHE  $\leq 3$  nM). In contrast to the hand-drawn binding curves, the simulations revealed no break. The predicted POB-treatment curve was also noticeably lower than the control curve at all concentrations of DHE, as expected after blocking of the high affinity class of binding sites, again in contrast with their experimental data.

Their second conclusion also contrasts with previously published findings<sup>4</sup> which indicated that POB treatment only reduced the binding capacity of the uterine  $\alpha$ -adrenergic receptors, leaving unchanged their uniform affinity for DHE. It has been established by several laboratories that at least two distinct types of  $\alpha$ -adrenergic receptor, termed  $\alpha_1$  and  $\alpha_2$ , can be identified in radioligand binding studies<sup>9-12</sup> and physiological studies<sup>13</sup>. DHE has been found to bind with indistinguishable affinity to both subtypes of receptor<sup>3,4,9,10</sup>. By contrast, phenoxybenzamine has been shown in some physiological studies to be  $\alpha_1$  selective<sup>13</sup>. Thus, the observation by Kunos *et al.*<sup>1,2</sup> of complete blocking of the  $\alpha$ -agonist response in the face of partial loss of DHE binding (Fig. 1, ref. 1) may be interpreted as the result of selective blockade of the  $\alpha_1$  subtype of DHE binding site. However, this conclusion cannot be substantiated by the DHE binding data that they provided (Fig. 2, ref. 1) and would require more quantitative binding studies involving  $\alpha_1$ - and  $\alpha_2$ -selective ligands such as prazosin and yohimbine, as presented elsewhere<sup>9</sup>.

In conclusion, inappropriate and erroneous interpretation of binding data have led Kunos *et al.*<sup>1,2</sup> to incorrect interpretations concerning DHE binding to uterine membrane preparations. In addition, the effect of POB treatment on DHE binding at higher ligand concentrations (8 nM) reported in Figs 1 and 2 (ref. 1) are internally inconsistent. All available data (including those of Kunos *et al.*) indicate that DHE binds to uterine  $\alpha$ -adrenergic receptors all of which show indistinguishable affinity for DHE.

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**KUNOS ET AL. REPLY**—In answer to the points raised by Roberts *et al.*, the definition of receptors involves a physiological function: our findings<sup>1</sup> dissociate the phentolamine-suppressible binding of high concentrations of dihydroergocryptine (DHEC) from  $\alpha$ -receptors mediating smooth muscle contraction. The low potency of phenoxybenzamine (POB) in suppressing DHEC binding was evident in earlier studies<sup>2</sup>; our findings confirmed and explained this discrepancy. As for the specific points raised: To their first point, yes, we should assume that, in the demonstrated absence of 'spare'  $\alpha$ -receptors in our system<sup>1</sup>, the potency of POB should be the same for block and for suppression of binding. Efficiency of coupling does not affect antagonists unless they act at a stage subsequent to receptors. It is also erroneous to suggest that block of responses to noradrenaline involves non-adrenergic receptors. Even if low concentrations of POB interacted with other receptors, this would be irrelevant to its effect on DHEC binding; even so, we found that serotonin and pilocarpine have no mechanical effects in rabbit uterine strips, and contractions by histamine are unaffected by  $3 \times 10^{-8}$  M POB, which blocks noradrenaline completely.

Concerning their second and third points,  $K_d$  values were determined in individual preparations but, due to the limited amount of membrane material, data points were concentrated either below or above 3 nM. If POB treatment is omitted, more control points can be obtained. Recently, we confirmed a small

break in the DHEC binding isotherm constructed from 18 data points at close concentration intervals. Scatchard plots of such curves are linear points below 3 nM and curvilinear with initial upward convexity above 3 nM; this is very similar to recent findings in the rat heart<sup>3</sup>. In this latter study, positive cooperativity between sites of the second binding component has been proposed to account for the curvilinear Scatchard plot. In the absence of sufficient numbers of closely spaced data points in the area of the break (2-4 nM) such deviations are difficult to identify, particularly if the linear sections of the plot have similar slopes. Also, we found that because DHEC is adsorbed to glass and many plastic materials, total ligand concentrations must be verified in aliquots of every assay tube to avoid errors in calculating bound/free values. In answer to the fourth point of Roberts *et al.*, the reason that an expected small suppression by POB at higher DHEC concentrations was not detected is not clear. It certainly could not be explained by reversal of the block by POB. The irreversible nature of the block of  $\alpha$ -receptor responses by POB is generally accepted<sup>4</sup> and this was also shown for block of <sup>3</sup>H-DHEC binding to uterine membranes in conditions identical to those used by us<sup>5</sup>. Direct evidence for the irreversible binding of POB to  $\alpha$ -receptors was recently provided in studies using <sup>3</sup>H-POB of high specific activity in intact liver cells<sup>6</sup> or liver membranes of rats<sup>7</sup>. It is possible that the expected 20-30% suppression of binding may have been obscured by the relatively large variation of values at higher DHEC concentrations (Fig. 2, ref. 1). In a different set of experiments, shown in Fig. 1, ref. 1, the same POB concentration ( $3 \times 10^{-8}$  M) suppressed the binding of 8 nM <sup>3</sup>H-DHEC by 27%. Finally, the ligand concentrations are not easily identifiable from the Scatchard plots shown<sup>8</sup>: in oestrogen-treated preparations there seem to be no more than three points or less than one point per preparation under 3 nM <sup>3</sup>H-DHEC.

The points raised by De Lean and Lefkowitz are the same as the third and fourth points of Roberts *et al.*, but they are also illustrated by computer analysis of our data. The inability of the computer to detect a break in our control binding curve is not unexpected; the  $k_d$  values for the two binding components were very close<sup>1</sup> and as the  $K_d$  of the second binding component was determined from points outside the area of the break (see Fig. 2 legend<sup>1</sup>), possible localised changes in binding affinity would not be reflected in the value obtained (see above). It is clear that on the basis of these data alone we should have disregarded the small break in our control curve, which is similar to the break evident in the DHEC binding isotherm reported by Williams *et al.* (Fig. 2, ref. 2). However, we could not have dis-

regarded the major difference in the ability of POB to block DHEC binding below and above the same concentration where the break in the control curve is apparent (Fig. 2, ref. 1). Such a differential effect of a suppressing ligand is only possible if the POB-sensitive and POB-insensitive sites have different DHEC binding properties. Failure of the computer to simulate our sigmoidal POB curve indicates that DHEC binding in the real tissue is more complex than the model on which the computer program is based. For example, positive cooperativity between sites of the second binding component is a possibility compatible with all our findings (see above), although a detailed analysis of the second binding component at different levels of receptor occupancy<sup>3</sup> was not possible due to the limited number of data points.

In agreement with our findings and in contrast to the above conclusions of De Lean and Lefkowitz, evidence in two recent studies also indicates binding of DHEC to two sites with differential affinity. In the rat heart ARC 239, an  $\alpha$ -receptor antagonist similar to POB in its postsynaptic selectivity<sup>3</sup> was also similar to POB in preferentially suppressing the binding of low concentrations of <sup>3</sup>H-DHEC<sup>3</sup>. In the rat liver, dissociation of 20 nM <sup>3</sup>H-DHEC had two components, indicating two binding sites with different affinities<sup>9</sup>.

Thus, our results<sup>1</sup> indicate the presence in the uterus of two DHEC binding sites, only one of which, preferentially labelled at concentrations <3 nM, is involved in  $\alpha$ -receptor mediated smooth muscle contraction. The exact nature of binding of DHEC to the second class of binding sites and the physiological function of these sites in the uterus remain to be clarified. Computer analysis of binding isotherms will give rise to erroneous conclusions unless the validity of the model used is firmly established.

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## Sensitivity of human bone marrow cells to cyclosporin A

FROM observations of the effect of cyclosporin A *in vitro* on committed progenitor cells of the myeloid and erythroid series (CFU-C and BFU-E), Gordon and Singer<sup>1</sup> have inferred that cyclosporin A has non-myelotoxic immunosuppressive activity. We find this a disturbing conclusion because although similar studies in this laboratory<sup>2</sup> using the semi-solid agar CFU-C assay<sup>3</sup> showed almost identical dose-related inhibition of CFU-C, our results also suggested that sensitivity to cyclosporin A is related to the level of differentiation of progenitors in the granulocyte-monocyte pathway, the more primitive progenitors being more sensitive. This is important because the pluripotent haematopoietic stem cell (CFU-S) from which CFU-C are derived and on which haematopoietic recovery in marrow transplantation depends, may be more sensitive to cyclosporin A than results of CFU-C assays indicate.

There is now considerable evidence that clone size potential decreases and sensitivity to factors with colony-stimulating activity (CSA) increases as progenitor cells differentiate down the granulocyte-monocyte pathway<sup>4,5</sup>.

Our experiments showed that: (1) cells forming small clones were less sensitive to cyclosporin A than cells forming large clones; (2) cells requiring the highest levels of CSA for clone formation (that is, the most primitive progenitors) were more sensitive than cells forming clones at lower CSA levels.

## Correlation between heart attacks and magnetic activity—a retraction

WE recently showed a significant correlation between the daily sums of Kp, the planetary index of geomagnetic activity, and the daily admissions of cardiac emergency cases to the two main hospitals in Hyderabad and Secunderabad<sup>1</sup>. Richard Peto of the Radcliffe Infirmary, Oxford, has re-examined our data, and finds that their distribution about the regression line is grossly sub-poissonian. This means that the correlation is higher than would be expected even if magnetic activity were the sole cause of heart attacks, and casts serious doubt on the authenticity of the data. The magnetic activity data are those published by the International Association of Geomagnetism and Aeronomy, and are above suspicion. The medical data are those used by Srivastava, Bhaskara Rao and Saxena<sup>2</sup> and were abstracted by colleagues of Srivastava (Bhaskara Rao, Saxena and two others) from individual case sheets and the hospital records. Srivastava and Saxena have re-examined

Both observations point to a trend of increasing sensitivity to cyclosporin A as the CFU-C/CFU-S transition is approached and thus to the possibility of considerable myelotoxicity in conditions where CFU-S are rapidly proliferating. Even without speculating on the sensitivity of CFU-S, our results for CFU-C detected at the highest CSA levels (approximately 50, 70 and 90% inhibition at 1, 5 and 10  $\mu\text{g ml}^{-1}$  of cyclosporin A, respectively) are disturbing, as 5–10  $\mu\text{g ml}^{-1}$  was required for near-maximal inhibition of the most sensitive T-lymphocyte colony-forming cell population in two of three patients studied by Gordon and Singer<sup>1</sup>.

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GORDON AND SINGER REPLY—The point that cyclosporin A may be considered to have some myelotoxicity *in vitro* is well taken. In terms of bone marrow engraftment, however, myelotoxicity has not been a problem. Cyclosporin A increases the survival of rabbits grafted

with allogeneic marrow<sup>1</sup> and is an effective prophylaxis against graft-versus-host disease in man<sup>2,3</sup>.

Francis and Berney have suggested that pluripotent stem cells (CFU-S) may be more sensitive to the compound than committed progenitor cells (CFU-G) and that cyclosporin A may have greater toxicity towards rapidly proliferating cells. These suggestions are based on the findings that small clones, grown in agar culture, are less sensitive to cyclosporin A than large clones and that sensitivity to the compound increases with the amount of colony-stimulating activity (CSA) presented to the treated cells.

Our data<sup>4</sup> do not show whether cycling and non-cycling haematopoietic progenitor cells differ in their sensitivities to cyclosporin A as the study used normal steady-state cells. This aspect is certainly of interest and experimental evidence can be obtained by studying the effects of cyclosporin A on patients' colony-forming cells following bone marrow transplantation.

The design of the experiments<sup>5</sup> said to show an effect of CSA activity on cell sensitivity to cyclosporin A involves a preincubation step before the cells are washed and plated in agar. It seems reasonable to suggest that the apparent effect of CSA activity on cell sensitivity may result from an altered responsiveness of the cyclosporin A-treated cells to CSA (or to inhibitors). Again, this can be tested by producing full dose-response curves of post-cyclosporin A cells, preferably to a cell-free source of CSA, which will give more reliable results than cellular feeder layers.

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the hospital records, which are now in a more accessible form, and are unable to reproduce the numbers abstracted earlier. We conclude that the medical data used in the two studies<sup>1,2</sup> are spurious, and any results derived from them should be disregarded. This may explain why Knox *et al.* have carried out a similar enquiry and failed to confirm our claims<sup>3</sup>.

We are grateful to Dr Peto for drawing our attention to this matter, and apologise for publishing a misleading result.

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## Glaciation due to continental drift

COGLEY<sup>1</sup> has suggested that a special distribution of land is needed during glacial periods. He proposes that the onset of glaciation occurs when land masses congregate in the tropics. The average global temperature decreases as the polar regions become "slightly warmer while

the tropics cool substantially". This is a consequence of the albedo contrast between land and water varying with latitude.

The physical situation is more complicated than Cogley suggests and it is possible that many mechanisms operate which can induce glaciation. Tarling reported an analysis of ice ages which suggested that a continental block is needed in polar areas<sup>2</sup>, especially in the Southern Hemisphere. Nevertheless, a near-polar position is insufficient to induce glaciation<sup>3</sup>, a point with which Cogley concurs. But his argument depends on variations in albedo contrast. Tarling maintains that adjacent seas are necessary to provide moisture for precipitation. In such conditions the mid-latitudes become sensitive to comparatively small changes in radiation such as those caused by variations in the Earth's orbit and the tilt of its axis<sup>2</sup>. However, contemporary theories of glaciation involve galactic mechanisms<sup>4-6</sup> only insofar as they influence the intensity of solar radiation. In what way can the reduction of solar radiation due to the Solar System passing through a dense galactic cloud (which has been shown to be related to the occurrence of ice epochs<sup>4</sup>) "modulate known tectonic mechanisms" here on Earth?

The distribution of the continents is evidently important. Oceanic current flow, cloud cover and atmospheric circulation, which ultimately determine the amount of heat absorbed, are all affected by continental drift. In the late Cenozoic era, Australia and Antarctica drifted apart, commencing around 55 Myr BP<sup>3</sup>. Circum-Antarctic oceanic circulation increased as the continental barrier was gradually removed with deep water flow occurring after the South Tasman Rise had subsided around 38 Myr BP<sup>3</sup>. After this time an increase in the extent of pack ice occurred with a profound effect on the Southern Ocean<sup>3</sup> and on polar albedo. Kennett also reports that equatorial oceanic circulation diminished during the Cenozoic because of the destruction of the Tethian Seaway and, slightly later, the blocking of the Indonesian Seaway.

On what grounds does Cogley assume that sea level has remained constant between 240 Myr and 0 Myr? Eustatic changes of up to several hundred metres have been proposed<sup>7-9</sup>. A quantitative assessment of changes of land area with latitude and with time cannot be made from maps of palaeocontinents delimited only by the present day shoreline<sup>10</sup>. For example, land areas on the North American continent in the late Cretaceous were at least 50% less than at present<sup>9</sup>, a fact that cannot be derived from palaeocontinental maps which do not show any variation of coastline with time. Using Cogley's own formula (equation 1) the change in land area of North America alone would produce an increase in the

average global absorbed radiation (about  $1 \text{ W m}^{-2}$ ) which is significantly greater than the variations he proposes through continental drift. Cogley states that the Mesozoic was warm, yet he presents a diagram of average global absorbed radiation over 240 Myr with a minimum (implying coolness) during the Mesozoic. If land area changes are taken into consideration, there should have been an increase in absorbed radiation.

Cogley should have considered the effect of clouds in more detail. Cloud plays a very important part in the global radiation balance<sup>11</sup> and determines the atmospheric transparency term in his equation 1. He has argued a dynamic model of glaciation from static components: from a snow-free world with contemporary cloud cover, from a world without changes of sea level and without pack ice. Albedo contrast may well play a part in initiating glaciation but the evidence cited does not support Cogley's thesis.

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**COGLEY REPLIES**—I take Reynolds' most important point, that my model is simple while the world is complicated. I do not claim that the model is a comprehensive theory of glaciation; I say merely that physical reasoning leads us to expect a palaeoclimatic signal due to albedo

contrast, and that if we could find it this signal would deepen our understanding of glaciation. Reynolds is entitled to reject my interpretation of Fig. 1c of my letter. He seems to have no quarrel with the concept of albedo contrast, but to claim instead that the signal is likely to be swamped by other effects. This would not surprise me in the least, and I look forward to accurate estimates of these effects.

Many palaeogeographic effects are consistent with my model. A tropical clustering of landmasses surely suggests reduced tropical, and enhanced higher-latitude, meridional circulation in the oceans<sup>1</sup>. Kennett's evidence for sea ice, found in Southern Ocean sediments after 38 Myr BP, is not in conflict with what I deduced from the concept of albedo contrast. Note that I made no attempt to model the transition to glacial conditions; by design, my radiative calculations were for a snow-free world without pack ice, a state of affairs for which the apparently almost complete absence of glaciogenic rocks in the Mesozoic<sup>2,3</sup> provides circumstantial evidence.

I am unable to comment further on galactic mechanisms; my use of the verb 'modulate' was unthinking.

I assumed constant sea level because I wished to proceed from the simple to the complex. Maps of ancient shorelines carry uncertainties of their own<sup>4</sup>, and the only global set<sup>5</sup> is 30 years old; although it was carefully compiled, it needs updating. One should be clear as to how continental flooding bears on my model. To nullify the concept of albedo contrast, flooding would have to be strongly biased towards either high or low latitudes, but such a bias is not seen. I fully agree that flooding may be important in its own right, and will affect my numerical results; I have indeed repeated the procedures of my letter for a series of equal-area maps based on the Termier and Termier set<sup>6</sup>, but I am not yet able to discuss these results.

I agree also that clouds deserve thoughtful attention, but I emphasise again that my extremely simple model remains physically sound. I suggest that the time is ripe for modelling Phanerozoic climates at finer scales of resolution, and in increasing detail. However, I would caution that efforts at improving accuracy<sup>6</sup> should keep pace with speculative insight.

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## Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.



## BOOK REVIEWS

## Changing views of man

Antony Flew

THIS slim volume consists of five of the six 1976 Herbert Spencer Lectures, a paragraph of Preface, and an Index. There are five only because the sixth lecturer, whom the Editor does not scruple to name, produced no script. No information about the contributors is provided, and there are more misprints — none of these sense-destroying — than one expects under the Oxford imprint. The Editor does not quote the terms of reference under which the lecturers worked. But, speaking presumably for the electors, he does say: "... we have gathered together a group of distinguished men from different disciplines to tell us how current work in their own subjects is changing their views of man".

The first essay, by Amartya Sen, is "Rational fools: a critique of the behavioural foundations of economic theory". The target in Sen's sights is that theoretical fiction, economic man; who is, by definition, "actuated only by self-interest" (page 1). But he seems not fully to appreciate how drastically this fiction is itself changed if you then proceed "to define a person's interests in such a way that no matter what he does he can be seen to be furthering his own interests in every isolated act of choice" (page 5). For a person who is thus necessarily in every choice striving — as the economists say — to maximise his own utilities may well be — in the everyday sense — acting very disinterestedly. What Sen explores is the huge area in which actual behaviour, though necessarily maximising the agent's utilities, is by no means reducible to the pure pursuit of that agent's self interest, as ordinarily construed.

In "Psychology and the image of man", Jerome Bruner confesses that in working on his lecture he was "drawn to a disturbing conclusion on the matter of why experimental or academic psychology had not had more of an impact on the broad cultural conception of the nature of man ..." (page 27). I now confess that, having read the resulting lecture, I am more than ever inclined to think that the reason is that it has had nothing to say that should be of general interest: the genuine discoveries have no wider implications; while the claims which do have a shattering impact are not supported by the empirical work. Skinner, for instance, denies the reality of human choice and human purpose. But these claims are what he mistakes to be presuppositions of any science of psychology. They neither are nor could be

*Scientific Models and Man: The Herbert Spencer Lectures, 1976.* Edited by Henry Harris. Pp.102. (Oxford University Press/Clarendon: Oxford, 1979.) £5.95.

findings of his own studies of rats and pigeons.

In "Memory and its models" J.Z. Young writes: "The concept that the brain contains information in a code is the new scientific model of memory, which I believe is revolutionising our view of man" (page 45). I am thus startled to be made to feel that I have all my life been a child of the revolution. For I cannot recall ever believing that information is stored in the brain in the form in which it is remembered. How could it be?

"Machine models of perceptual and intellectual skills" by Donald McKie is fascinating stuff: "The world has a false belief about calculating prodigies, namely that they calculate prodigiously" (page 74). The truth is, apparently, that they use all manner of calculation-saving dodges. The same is true of chess-masters: they neither do nor could work through every thinkable alternative many moves ahead. To achieve a Grandmaster level of play by a computer program on these lines: "It would ... take of the order of  $10^{90}$  years' continuous running on a super-fast machine to select a move" (page 77). For the benefit of possible philosopher-readers

of *Nature* I have to mention the suggestive treatment of the seventeenth-century Problem of Dr Molyneux: "R.L. Gregory studied just such a case, in which the patient had had sight conferred on him by an operation of corneal grafting" (page 62).

In excellent taste, the most exciting contribution is kept till last. In "The universe as artefact", D.H. Wilkinson ponders Einstein's homespun question: "What does a fish know about the water in which he swims all his life?" (page 80). Wilkinson's ponderings raise questions about ways in which the basic facts about our nature — size, length of life-cycle, and so on — must eventually limit our possible physical knowledge: "... already in the big sciences it takes typically 5 years from inception to completion of an experiment ... When we get to 100-year long experiments which have to be passed from academic father to academic son we shall not have many takers ... " (page 82). But I wish that Wilkinson had ended, not by first mentioning and then repudiating Berkeleyan idealism, but with his earlier words: "Perhaps the impossibility of finding quarks is Nature's way of letting us know that we have reached the end of the line." But we know that we could not accept that." (page 97). □

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## Crossbridge theory in the dock

R.M. Simmons

*The Molecular Basis of Force Development in Muscle.* Edited by N.B. Ingels. Pp. 162. (Palo Alto Medical Research Foundation: Palo Alto, California, 1979.) \$17.

THE sliding filament theory of muscular contraction is generally accepted as well established. Less certain is the mechanism of force generation and shortening, but the great majority of workers in the field accept some form of crossbridge mechanism at the very least as a working hypothesis. There are dissidents from the majority view. This book, the proceedings of a conference held in Miami in 1977, is edited by one of them and the

conference evidently served as a platform for the views of another (Pollack). Perhaps court-room would be more appropriate than platform, for Pollack seizes the roles of prosecutor, judge, jury and would-be executioner, with orthodoxy embodied by H.E. Huxley, Podolsky, Weber and Julian in the dock.

The trial opens with short pleas from the defendants. The case is nicely argued on the grounds of structure (Huxley and Podolsky), biochemistry (Weber) and mechanics (Julian and Podolsky). The prosecution case from Pollack, subsequently enlarged on in a lengthy cross-examination of the defendants, involves two main charges. The first is that the constancy of filament length demanded by the sliding filament theory is not always to be found in the literature, but Pollack's attempt to weigh the evidence by number of pages rather than by scientific merit becomes more of a trial of patience than of fact. Huxley defends the case as

trenchantly as he did twenty years ago and the theory emerges from the trial without much of a stain on its character. The second charge concerns the length-tension relationship, one of the corner-stones of the crossbridge hypothesis. Gordon, A.F. Huxley and Julian showed in 1966 that the tension developed by a muscle fibre depends linearly on the overlap between the filament, though if stimulation of stretched fibres is prolonged tension continues to rise slowly. This was ascribed to the shortening of some of the sarcomeres. Pollack states that he does not observe such a shortening, at least using light diffraction. On the other hand Julian says he does, using photography. A fair verdict would be that the charge is "not proven."

Ingels makes what seems to be his second contribution to the field with a chapter on "Unifying Concepts" which attempts to unify his first contribution, a non-

crossbridge theory, to crossbridge theory, but little else. As organiser of the conference he must bear some responsibility for the fact that nearly two-thirds of the book is unrepresentative of current research in the field. The chapters on crossbridge theory and practice might serve as an introduction to the field and contain some new results particularly from X-ray diffraction studies, but they are too brief. The discussion never gets away from the two issues reported above. The question of whether current results from structural, biochemical and mechanical studies can be explained on other than a crossbridge model is not raised. There is an Appendix from Ingels with a chronology of muscle theories, some of which are splendidly batty. □

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## Molecular basis of tumourigenicity

Dennis D. Cunningham

*Surfaces of Normal and Malignant Cells.* Edited By R.O. Hynes. Pp.471. (Wiley: Chichester, UK, and New York, 1979.) £24.

THE cell surface has become a prime target for studies on the molecular basis of tumourigenicity. In a lucid introductory chapter, the editor summarises reasons for this, discusses issues basic to such studies (for example, do properties of transformed cells in culture accurately reflect the properties of malignant cells *in vivo*?), and sets the stage for the subsequent contributions.

A chapter on morphology of normal and transformed cells is followed by three chapters on cell surface composition which emphasise glycolipids, proteins and glycoproteins, and mucopolysaccharides. Two chapters focus on enzymes of normal and transformed cells, and there is a chapter on immune responses to C-type virus-induced tumours. From here on, the chapters mostly deal with control processes in normal cells: early events in growth stimulation, surface transport components of erythrocytes, adhesive specificity among embryonic cells, and the cell surface and development in the cellular slime mould *Dictyostelium discoideum*.

A theme which recurs in this volume is that the several properties which appear to correlate generally with the transformed state are not invariably found in all transformed or tumourigenic cells; it is probably certain combinations of these, along with alterations yet to be defined, that collectively underlie malignancy.

Given present uncertainties about which of the many alterations found in transformed cells and their surfaces might be causally related to malignancy, and the fact that any given topic on regulation of cellular activities could relate to alterations basic to malignancy, the editor faced a real challenge when choosing the issues to be addressed in this volume.

Although most of the choices were excellent, it would have been an easy matter to justify individual chapters on assays for tumourigenicity (malignancy?) of cultured cells, and the use of melanoma lines with specific metastatic potentials to probe cell surface properties related to metastasis. As emphasised by the editor, the commonly

used assays for tumourigenicity of cultured cells depend on growth and survival of the cells and not generally on their ability to invade and metastasise, even though the latter two properties distinguish malignant tumours from ones that are benign.

As "a mere collection of data would soon become out of date . . . the authors were encouraged to extrapolate from the established facts and discuss hypotheses concerning their significance". As a result, the individual chapters are generally conceptual in nature, and provide the reader with interpretations of results. Thus, the volume does what this type of book should: synthesise and extrapolate rather than present previously published data. At the same time, each chapter is quite well referenced, providing the reader with ready access to the original literature. In general, the individual chapters are well written and the authors emphasise questions that remain to be answered. Two chapters, "Surface Membrane Enzymes in Neoplasia" and "The Cell Surface and Development in the Cellular Slime Mould *Dictyostelium discoideum*", were disappointing. Both were rather long on facts and short on interpretation. Unfortunately, the latter chapter did not contain a much needed summary section.

My overall impression of this book is quite favourable. It generally emphasises ideas and questions; at the same time, it contains enough experimental facts to be informative and credible. With exceptions in only several parts, the book is well integrated, a feature which should increase its general appeal. □

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## Wave functions for atoms and molecules

A.D. Buckingham

*The Calculation of Molecular Orbitals.* By J.C. Slater. Pp. 104. (Wiley: New York and Chichester, UK, 1979.) £11.50; \$23.

PROFESSOR J.C. Slater was a theoretician of the first rank and a prodigious writer of textbooks. The present work is the last in a series of eight books by Slater on the quantum theory of matter (the first was published in 1960). The manuscript of this book was found among his papers following his death in 1976, the only addition being a list of a small number of references.

In the last ten years of his life, Slater was concerned with the local-density form of the self-consistent field; his work led to the so-called  $X\alpha$  method, and he and many

others applied it to a wide range of problems in theoretical physics and chemistry.

This new book of only a hundred pages centres on the SCF  $X\alpha$  method for obtaining approximate electronic wavefunctions for atoms and molecules.

Applications of the  $X\alpha$  method have involved approximations which impose limitations on the validity of the results. Slater's purpose in writing this book was to suggest a different scheme for solving the SCF equations. He turned to the cellular method, developed by Wigner and Seitz for handling the problem of computing the orbitals of a crystal. (Slater himself was a pioneer in this energy-band theory.)

All those who are interested in the SCF  $X\alpha$  method will find this a most useful and informative book. It is the writing of a mature scientist, one of the masters of theoretical physics and chemistry. □

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# Single-stranded DNA phages

Mary R. Lunt

*The Single-Stranded DNA Phages.* Edited by D.T. Denhardt, D. Dressler and D.S. Ray. Pp.720. (Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1979.) \$49.

It is now twenty years since Sinsheimer wrote that finding a single-stranded DNA molecule was 'like encountering a unicorn in the ruminant section of the zoo'. Since then studies on the single-stranded DNA phages have not only produced information relevant to fundamental biological processes but have contributed much to the development of methods now routinely applied in protein and nucleic acid biochemistry.

This book arose from an international meeting held in August 1977, but includes references to papers and unpublished work up to 1978. The articles are comprehensive and well-written reviews of all aspects of the biology of the single-stranded DNA phages. They include discussions of the structural and regulatory roles of the protein-nucleic acid interactions involved, thus providing interest well beyond the specialist areas considered.

The papers mainly refer to the isometric phages  $\phi$ X174, S13 and G4, and to the male-specific filamentous phages f1, fd and M13. They all use *Escherichia coli* as host and have virions which contain single-stranded, covalently closed, circular DNA of molecular weight about  $2 \times 10^6$  and coding for nine genes. Besides being the first virus shown to contain such DNA,  $\phi$ X provided the first example of overlapping genes, that is, regions in which the DNA codes for more than one protein, the RNA transcripts being read in different phases. There are articles giving the complete nucleotide sequences of  $\phi$ X, G4 and fd DNAs, and discussions of the gene assignments and gene functions. The filamentous phages do not have overlapping genes, and can package variable amounts of DNA, as described in the use of M13 as a cloning vector.

The chapters on DNA synthesis review the many elegant experiments on the structures of the replicating forms, and their reproduction *in vitro*. The general significance of these studies is apparent in their analysis of the probable roles of host proteins in bacterial DNA synthesis, and also in their revelation of the importance of DNA secondary structure and its modification by enzymes such as DNA gyrase, helicases and the *E. coli* rep protein.

The analysis of transcriptional control of protein synthesis by these phages has revealed their ability to make a variety of polycistronic RNAs in which sequences

coding for proteins made in large amounts appear in more than one RNA species. There are discussions of the structures recognised by RNA polymerase at initiation and termination sites, now made possible by the availability of DNA sequence data.

Increasing attention has recently been given to the formation of intact virions. The isolation of  $\phi$ X precursor particles is described and the protein-DNA interactions are discussed. The intriguing production of filamentous phage particles without cell lysis now seems to present an example of the 'signal' mechanism, as nascent coat protein molecules are probably embedded in the cell membrane by way of a leader sequence, the final protein product exchanging with the intracellular DNA binding protein as the

phage DNA is extruded from the cell.

The chapters on filamentous phage virion structure provide detailed discussions of diffraction and other data which support different models for the spiral arrangement of protein monomers around the central DNA. There are also accounts of the use of single-stranded DNA phages in the analysis of genetic recombination, and of the disruption of virion structure which occurs when  $\phi$ X particles adsorb to the host cell.

The book is not for beginners, but it contains much which can be appreciated and enjoyed by advanced undergraduates and those who teach them. □

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## Vitamin D metabolism and function

R.H. Wasserman

*Vitamin D.* By H.F. DeLuca. Pp.80. (Springer: Berlin, New York and Heidelberg, 1979.) DM29; \$15.

VITAMIN D has long been appreciated as the primary factor required for the prevention and cure of rickets and osteomalacia in man and animals. This factor, acquired exogenously or synthesised endogenously by the skin upon exposure to UV light, undergoes biochemical transformations to yield various metabolites, including those hydroxylated in the 25 position [25(OH)D], in the 1 and 25 positions [1,25(OH)<sub>2</sub>D] or in the 24 and 25 positions [24,25(OH)<sub>2</sub>D]. The 1,25(OH)<sub>2</sub>D has recently become recognised as a bona fide hormone since it affects reactions elsewhere than at the site of synthesis (kidney) and is under feedback control.

The biological effects of vitamin D and its metabolites have been extensively studied in recent years, and a considerable amount of new information and concepts on this aspect have also become available recently. Among these are the protein synthetic theory of vitamin D action, the identification of several proteins induced or modified by steroid repletion, the interaction of metabolites with the intestine, kidney and bone, and with organs not traditionally considered to be targets, such as the parathyroid gland and pancreas, and details on the translocation of calcium and phosphate across epithelial membranes, to mention just a few.

H.F. DeLuca has gathered together and integrated in a short monograph much of

the recent advances. Specifically discussed in this volume are historical aspects, the chemistry of precursors and metabolites of vitamin D, the biological control of the formation of vitamin D by nutritional and hormonal variables, the molecular events affected by vitamin D that bear on calcium and phosphate metabolism, and therapeutic experiences with vitamin D and its metabolites.

Of considerable value are the critical analyses by the author of present concepts and of various published studies. Areas in which there is a paucity of information, and where controversy abounds, are frequently noted. However, not all experts in the field will agree with some of the interpretations and viewpoints of the author, perhaps an expected spin-off of a very active research area. For this reason, the reader is urged to consult other recent reviews and monographs on this topic in order to obtain a broader perspective.

The monograph is well written, lists about 400 references, and is reasonably well illustrated. Since the author and his associates around the world have been prolific contributors to vitamin D research, this monograph in a sense represents a cataloguing of their many contributions (about 30% of the references cited are authored or co-authored by Professor DeLuca).

This monograph should appeal both to the specialist on vitamin D, calcium and phosphorus metabolism, and to the interested non-specialist. It should also prove useful to the clinician, providing an introduction to the chemical and biological behaviour of a class of compounds that are efficacious in some disease states and potentially therapeutic in others. □

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## Basic biology of *Drosophila*

Peter Lawrence

*The Genetics and Biology of Drosophila*. Vols 2a, 2b and 2c. Edited by M. Ashburner and T.R.F. Wright. (Academic: London, New York and San Francisco, 1979.) Vol. 2a: £28.50, \$59; Vol. 2b: £29.60, \$61.25. Vol. 2c: £32, \$74.

ASHBURNER and Wright is a mammoth read. Or is it? This is the question for the *Drosophila* worker: Are the three parts of this volume a handbook for reference or a collection of ephemeral reviews for reading? If the former then the *Drosophilist* will want to buy them (or, rather, prefer to beg or steal them as they cost nearly £90). If the latter, then a lending library can be relied on and the relevant chapters read. It is not clear what the editors would have their books be. They write that it is an attempt "to gather together as much information on the basic biology of *Drosophila* as possible excluding only its formal genetics found in volume 1 and its population biology to be covered in volume 3".

I think these books deserve to be judged by high standards and to me that means as a handbook, a book which no *Drosophila* worker can be without because it contains the basic facts and methods. A handbook should make accessible the present state of hard knowledge, where the literature is critically distilled and not merely catalogued. This is especially true with *Drosophila* where an indexed bibliography is available. Just as important, the methods, hints, tricks and ploys should be given special emphasis. In brief, the chapters of a handbook should be written with the readers' needs in mind. By contrast, in reviews, the author is frequently thinking of making some kind of a sale.

As these three books have, necessarily, been written by many authors, it is no surprise that this purpose is not achieved by all, or even by most. Some chapters are, and will be, extremely useful — others are already out of date or just forgettable. The useful or essential chapters are mixed up with the relatively useless ones; the topics are also randomly assorted. This means, of course, that we have to buy each volume even if we need only a small part of each. For example, to obtain the critical review on chromosome puffing the cytologist has to pay for what is in effect a 150 page monograph on behaviour — this topic is unlikely to be close to his, or her, heart. One wonders whether this is one reason why big multiauthor books are becoming so popular with publishers — a small number of desirable pages can sell a larger number of undesirable ones. Always true

of journals but not, in the good old days, true of books.

As I read through volume 2 it was not always easy to discern editorial purpose. The chapters are often of profligate and arbitrary length. Why, for example, should we have the same number of pages (100) on mutants which produce tumours (at the moment a somewhat unclear and peripheral subject for most *Drosophilists*) as on the central technical matter of chromosome aberrations (Volume 1)? Why so much on the field of imaginal disc development and its genetics? This field is interesting, but it is moving just too fast to be reviewed at such length in a handbook. Thus, because of long publication delays, the chapters on this topic are already becoming obsolete (from the literature references I get the impression that they were written about 3 years ago). By contrast, the scanning electron microscope atlas of the fly will be useful to many and retain its usefulness for years. The collation of culture methods is admirable and here one can detect the special atmosphere of free exchange of know-how and stocks, which

is such a pleasing aspect of work with the fruitfly.

In these books there are chapters on all manner of things: culture methods, both of flies and cells; nutrition; enzyme genetics; visual physiology; behaviour; tumours; chromosome puffing; the blood system; muscles; the fat body; malpighian tubules; ageing; embryonic development; imaginal discs and transdetermination. This means that every fly worker will want to read some parts and others will need to have the books within easy reach of their microscope. However, one cannot help feeling how much better they could have been. The chapters could have been more logically grouped. Ephemera could have been more conscientiously avoided. If the authors had been limited to numbers of pages which correlated with the importance of their subject, could this volume not have been at once shorter, cheaper and more valuable? □

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## Mechanisms of cell killing by radiation

J.H. Hendry

*Cellular Radiobiology*. By Tikvah Alper. Pp.320. (Cambridge University Press: Cambridge and New York, 1979.) Hardback £18; paperback £5.95.

THIS book would be more aptly titled '*Mechanisms in Cellular Radiobiology*' or more specifically '*Mechanisms of Cell Killing by Radiation*'. Written by one of the leading and often controversial figures in this field for a quarter of a century, the book traces in depth, both historically and scientifically, our knowledge of how various radiations cause a loss of reproductive integrity of cells when irradiated in different conditions.

It is the only book in recent years to compare and contrast fully and with clarity the response of different cell types from bacteria to mammalian cells, and it is this comparison which has provided much of our insight into the mechanisms of radiobiological effects. As the latter have not yet been fully elucidated, this has prompted hypotheses such as target theory and repair models to 'explain' dose-response relationships. These are discussed at length both in the first few and in the last few chapters. Sandwiched in between are the observations concerning dosage modifications caused by radiosensitisers

(primarily oxygen), radioprotectors, ionisation density, phase of the cell cycle at which cells are irradiated, dose fractionation, and dose-rate effects.

The failure of target theory to accommodate certain critical radiobiological findings has led to the clear conversion of the author from target theory to repair-type models which are less rigid in their constraints. Perhaps as a consequence the correlation between chromosomal injury and cell lethality, now convincingly demonstrated, receives little attention. The treatise of repair-type or pool models is novel and comprehensive.

The book concludes with a chapter on measurements of cell survival *in vivo*, which is purposefully concise but which is consequently not as comprehensive as the other chapters. Also, in a few instances, effects are discussed which have not generally been found by other investigators, for example, aspects of survival and dose-rate effects in bone marrow.

Although there are many useful tables of collated data, this is clearly not simply a textbook for learning by rote. It is more a stimulating thesis to be digested, argued, even attacked, and will undoubtedly remain a firm favourite with mechanistic radiobiologists. In contrast to nearly all other specialised texts, the provision of a separate paperback edition puts this volume within everyone's pocket. □

J.H. Hendry is Head of the Department of Radiobiology in the Paterson Laboratories at the Christie Hospital and Holt Radium Institute, Manchester, UK.



# OBITUARY

## Peo Koller

P. C. KOLLER, Professor of Cytogenetics at the Institute of Cancer Research, died on 29 June 1979. He will always be remembered by those who knew him well. For some it will be his splendid humour that endeared him, for others his fascination for the facts of life.

Born in 1904, he was taken as a novitiate into a Benedictine community at the age of twelve with the intention that he should eventually become a teacher in a subject that he found pleasing. Choosing botany and zoology he eventually graduated from the University of Budapest in 1924 at the age of 20. During his time in the monastery and university he had had to combine religious and scientific studies and it is clear from what he wrote in his unfinished autobiography that there arose in his mind, even before he graduated, some difficulties of reconciliation of the two disciplines. Despite this he was ordained into the priesthood the same year he gained his BSc.

Rather to his surprise his superiors offered him a teaching post in Budapest and encouraged him to undertake a PhD. He met a number of scientists including Voronow, Rhoda Erdmann and Kammerer at an International Conference in Budapest in 1927 and became committed to a research career. Shortly after joining the new hydrobiology unit at Tihany on Lake Balaton he was granted a scholarship to go to Cambridge in the United Kingdom.

This was the beginning of his break with the church which was in a large part due to increasing interest in genetics. As he explained it, the determinist philosophy of genetics, with which he became involved under the influence of his collaborators T.H. Morgan, Theodore Dobzhansky, C.D. Darlington and H.J. Muller, was the deciding factor. His interests turned specifically to cytogenetics which was a newly emergent discipline in the early 1930's. During and immediately after the war he became involved with the effects of alkylating agents, such as nitrogen mustard, on chromosomes, an interest which remained for much of the rest of his working life. He joined the staff of the Royal Cancer Hospital and soon afterwards its pup, the Chester Beatty Research Institute.

Peo's study of chromosome breakage in tumours particularly under the influence of cancer chemotherapeutic agents established him as a leading authority in the field. At that time, however, further and more exact developments of cytogenetic studies of mammalian somatic chromosomes was somewhat hampered by the inadequacies of the available methods.

In 1956 under the influence of Alex Haddow he became interested in the newly discovered radiation chimaeras, the analysis of which was best accomplished by cytogenetic methods with which Peo was familiar and which, largely under the influence of Peo's friend, Charles Ford, were now more reliable.

Peo's curiosity led him with enthusiasm into the fields of haematology and immunology which emerged as integral parts of the study of chimaerism.

He was a great biologist and always had the ambition that biology should be accepted in its own right as a major discipline in cancer research. In his latter working years he provided encouragement for a number of young research workers including J.F.A.P. Miller who made his discovery of the function of the thymus whilst under Peo's umbrella.

He rarely scolded his juniors and always provided the quiet and unobtrusive support which is so successful and so difficult to emulate. It should not be thought that he lacked in passion. His occasional anger was a source of amazement and delight to his colleagues who, on one occasion at least, had to shelter from bombardment with the bottles in which *Drosophila* flies were kept.

Peo found great happiness in his marriage and his three daughters were a constant source of delight and pride in his life.

After retirement he became an articulate and much-respected member of the rural community in which he and his family chose to live. His preaching there of the delights and method of biological science was probably in many ways the happiest time of a very full life.

He will be remembered with affection and gratitude by his friends, his colleagues and his neighbours. There was rarely a dull moment with Peo about.

Tony Davies

## C.E. Wynn-Williams

DR WYNN-WILLIAMS, a pioneer in the use of radio valves, valve amplifiers and thyatrons for measurement and counting in the fields of nuclear and radiation physics, and in the basic techniques of electronic computers, died at his home near Aberystwyth on 30 August 1979, aged 76 years.

He was born in Swansea on 5 March 1903, attended Wrexham Grammar School and entered the University College of North Wales at Bangor in 1920 with an Open Scholarship and a County Exhibition. He received the R.A. Jones

Mathematics prize in 1922 and graduated BSc Wales with first-class honours in physics in 1923. From 1923 to 1925 with the aid of a Research Studentship of the University of Wales he carried out research at Bangor under Prof. E. Taylor Jones, obtaining an MSc Wales in 1924.

He then continued with research in physics, at the Cavendish Laboratory, Cambridge, under Sir Ernest Rutherford, with, in succession, a University of Wales Open Fellowship, an 1851 Senior Fellowship and a moiety of the Clerk Maxwell Studentship. During this period he took a BSc London with first-class honours and received a Cambridge PhD in 1931.

His first two papers relating to valve amplification described a valve amplifier for ionisation currents with compensation for L.T. and H.T. battery fluctuations and equivalent to a quadrant electrometer of sensitivity more than 6000 mm per volt; and with the modifications of this necessary to allow it to be applied for measuring X-ray and photo-electric effects. He next worked in collaboration with Cave and Ward on the rate of emission of  $\alpha$ -particles from radium, and with Rutherford and Ward on the  $\alpha$ -rays from RaC, ThC and AcC, his main contribution being the design of the valve amplifier for the detection of the individual particles and its screening from outside effects.

A visit to the Cavendish Laboratory by A.W. Hull about the time of Hull's full description of thyatrons in 1929 stimulated Wynn-Williams' interest in them and led to his first step in their applications to counting when he devised a ring of four thyatrons and applied it in 1931 to further  $\alpha$ -ray counting experiments with Rutherford and Lewis, in the investigation of long-range  $\alpha$ -particles from ThC and AcC.

In the following year he published his "scale of two" thyatron counting system which, he pointed out, could be operated by current taken from the ordinary A.C. mains supply and required no delicate adjustments; it was this invention, followed six years later by a somewhat similar counter devised by W.B. Lewis employing hard valves instead of thyatrons, from which has mushroomed the great technology of digital electronics. In recognition of this work Wynn-Williams was awarded the Duddell medal of the Institute of Physics in 1957.

During the second world war Wynn-Williams was deeply involved in the work of code-breaking machines, in particular on the "Enigma" cipher machine and on the electronic side of the "Heath Robinson" cryptanalytic device which was

a step towards the later wholly successful "Colossus" machine. It is believed that he was at least in part responsible for the securing of T.H. Flowers and his team from the Post Office Engineering Research Station at Dollis Hill for the cipher work at Bletchley Park.

After the war, Wynn-Williams returned to Imperial College and threw himself whole-heartedly into teaching work in practical physics in which his great inventive skills and warm personality led to outstanding success.

The author of this obituary experienced these personal qualities to the full during the three years in which he was associated with Wynn-Williams at Cambridge and cannot speak too highly of him as a co-worker and friend, and for his help and encouragement.

F.A.B. Ward

## Vladislav Kruta

MANY physiologists and historians of medicine in France, Great Britain, Czechoslovakia, and as far afield as New Zealand will be sorry to hear of the recent death of Professor Vladislav Kruta, MD, DrSC, past director of the Institute of Physiology of the Purkyně University in Brno, Czechoslovakia.

Born in 1908, his scientific career started in the early thirties at the Department of Physiology of Charles' University in Prague. From the beginning his interest was focused on the comparative physiology of heart muscle and on the effects of temperature on contractility. His research on the "Bowditch staircase" became a classic. His work brought him into close contact with French physiologists and especially with the school of Professor Lapique, where he spent a long period and where he also met his future wife. These contacts gave Czech physiologists access to the French and Western scientific forum: as a General Secretary of the Czechoslovak Biological Society he made this society a branch of the old and famous French Société de Biologie.

As a convinced democrat, he left Czechoslovakia before the Second World War, spent a difficult period in France and was transferred after the fall of France with the Czechoslovak troops to Great Britain. As a medical officer, he became attached to the famous 312th Squadron of the Royal Air Force, composed mostly of Czechoslovak airmen, a squadron which won its glory during the Battle of Britain, with the loss of many lives. For his courageous conduct, Kruta was awarded one of the highest Czechoslovak military medals. Later in the war he was transferred to the Ministry of Social Affairs of the Czechoslovak Government in exile (in London) where he made use of his vast knowledge of the problems of nutrition to prepare the way in which the assistance of UNRRA should be made use of in his

liberated home country. Czechoslovakia owes him gratitude for the generous contribution of UNRRA in reopening and restarting medical and biological research after the war.

In 1945, Kruta became one of the founders of the new medical school in the city of Hradec Králové. As with many other people who fought in the Allied forces in the West, he had been persecuted since 1948 and dismissed shortly afterwards from the Hradec Králové medical school, when it became a military medical academy under Russian supervision. He then took over the Chair of Physiology at the Purkyně University of Brno. In spite of many obstacles of a political nature he created there an excellent modern school of physiology, concentrating mostly on experimental cardiology.

His classical education and early interest in general history led him later to the history of medicine and mainly to the two great Czech protagonists of physiology — Procházka and Purkyně. It was Kruta's great achievement to continue and complete the edition of Purkyně's *Opera omnia*, started before the War by the Prague histologist Prof. Studnička, and to write a monograph on both these great figures.

During the Prague Spring in 1968, Kruta deeply engaged himself in the democratization of life in Czechoslovakia. He was one of the 70 original signatories of the manifesto of "2000 words" and after the Russian occupation, and in spite of pressure, he refused to recall his signature. This led to his immediate dismissal from the chair and from all academic activities. As a private person he continued his historical studies. His courageous and honourable life made him a lasting model to his compatriots and friends.

Jan Brod  
Otokar Poupa

## Robert Heizer

ROBERT FLEMMING HEIZER, who died on 16 July 1979, at the age of 64, was one of the best known and most outstanding archaeologists to have been trained and who taught at the University of California at Berkeley.

His contributions to his profession, to the scholarly world, to the public in general and to his University were continuous and at an extraordinary and prodigious rate and level of creative excellence that is rare, even in the most distinguished communities of scholars. He was the author of 24 books and nearly 400 scientific articles and monographs that express the remarkable breadth and wide-ranging interests of California's pioneer scientific archaeologist who believed that it is impossible to understand mankind or to formulate "laws" of culture without using the data of prehistorians.

Ranging from book-length treatments of the Olmec civilisation of Meso-America or the prehistoric rock art of California and Nevada (on which he was the acknowledged authority) to a study of prejudice and discrimination against Californian minorities under Spanish, Mexican and US regimes, or X-ray fluorescence analysis of obsidians and replicative experimentation in archaeology, his work also included excursions into such subjects as toxicology, geology, mineralogy, organic chemistry, pedology, demography, mathematics, architecture, history, limnology, law, conchology, zoology and economics, wherever he believed they could throw light on past human behaviour. His investigations at La Venta, not far from the Gulf of Mexico, as well as in the Valley of Mexico and Guatemala led him to develop a keen interest in the transport of megalithic monuments and the methods of erecting them which resulted in a brilliant piece of research on the origins of the Colossi of Memnon on the Nile.

The quantity and quality of his research and publication may in time tend to obscure his work and ability as a teacher. In this, however, he was equally successful and an inspiring lecturer at all levels of instruction. His teaching ranged from North American and Meso-American archaeology to California Indians, archaeology and society, and science in archaeology and he was equally as successful in field courses as he was in the classroom and in seminars. He was always available to talk with students, especially with any who came to discuss their original ideas.

Heizer's work is both the foundation on which systematic archaeology in California has been based and a contribution of major significance for the science of archaeology in general. This was recognised in the honours conferred on him: he was elected to the Society of Sigma Xi, was twice a Fellow of the Guggenheim Foundation, a Fellow at the Center for Advanced Study in the Behavioral Sciences at Stanford and was elected a member of the National Academy of Sciences in 1973. He received an honorary ScD from the University of Nevada in 1965.

Bob was the kind of scholar who will not be seen again for a long time. He was a great archaeologist to whom prehistory in the western states will forever be indebted. He was possessed of a rather rare sense of humour and could be extremely witty and good company. Critical of others, he was equally critical of himself and in some ways was a "loner". His friends remember him for his humour, kindness and generosity, his students for the way he shared with them the excitement of new discoveries in the field and colleagues and students alike for the magnitude and depth of his scholarship and for the stimulus that he gave to the discipline in general.

J. Desmond Clark